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Orientador | Professora Doutora Ana Maria Ferreira de Sousa Sebastião

Dissertação especialmente elaborada para obtenção do grau de
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A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 19 de Fevereiro 2019

Agradecimentos

Quando decidi enveredar por este caminho, a minha ideia do que seria escrever uma dissertação era de que se trataria de um projecto inerentemente individual: a *minha* dissertação, o *meu* trabalho, a *minha* carreira. Aliás, estaria a mentir se não dissesse que essa foi uma das características que mais me atraiu acerca desta aventura. É por isso que agora, pouco mais de um ano depois de ter começado este trabalho, me encontro positivamente surpreendido com o quão *errado* estava. Apesar de ser o meu nome que está na capa desta dissertação, dizer que este trabalho é apenas meu não seria nada senão falso: este trabalho é tanto meu quanto das inúmeras pessoas que me ajudaram – directa ou indirectamente – ao longo dele, e sem as quais não o teria conseguido levar a bom porto. E embora tenha de agradecer a todos os membros do laboratório da Professora Ana Sebastião, pois cada um contribui da sua maneira única para fazer do laboratório o quão especial é, algumas pessoas merecem um destaque especial, pelos papeis que tomaram na minha vida.

Acima de tudo tenho de agradecer à minha orientadora, a Professora Ana Sebastião, por não só me ter recebido no seu laboratório, como por me ter aceite enquanto mestrando, e me ter dado a liberdade para fazer este trabalho algo mais meu. Mais ainda, não existem agradecimentos suficientes pelas oportunidades que me deu ao longo deste ano, e pelo tempo e disponibilidade que teve para discutir o projecto. Espero ter sido um bom mestrando e ter superado as suas expectativas para este trabalho.

Aos *Mizeses* devo, também, um obrigado colectivo – por me aturarem diariamente – e individual: ao João Gomes, por me ajudar com os *blots*, pelas conversas e momentos de brincadeira na nossa sala, pela variada banda sonora diária (não sei como te lembras de tantas músicas) e por me dar a perspectiva de que, se calhar, "isto" – seja lá o que "isto" for, em qualquer dia particular – não é o fim do mundo. À Rita Belo, lorde das bolhas, pela energia incontrolável, pelas conversas de café e os comentários aleatórios que nunca falham em me fazer rir, pelos conselhos científicos (e não só), e por ser uma das pessoas mais únicas que já tive o gosto de conhecer. À Sara Tanqueiro e à Catarina Beatriz, os meus putos, por me fazerem rir diariamente, por me darem conversa quando estou aborrecido, e sem as quais os dias no laboratório seriam muito menos felizes – isto sem vocês não era a mesma coisa. À Catarina Lourenço, por ter sempre uma resposta e um auxilio prontos às minhas dúvidas básicas, e me fazer companhia – com conversas sempre interessantes – durante tantos fins de semana no laboratório. E à Jéssica Rosa, que começou isto comigo,

e com quem as conversas são uma constante caixa de boas surpresas, sem as quais a nossa sala não seria o que é. Devo também um enorme obrigado à líder dos *Mizeses*, a Professora Maria José Diógenes, pela ajuda crucial com a obtenção do THC, e por não só juntar à sua volta um grupo de tão bons alunos, como por ser um modelo de entusiasmo e curiosidade incansáveis (e, já agora, por me tentar casar).

À Nádia Raquel, minha dama do sinal e vítima favorita, não só pela ajuda com as dissecções mas, mais que isso, por ser a (pequena) pessoa fantástica que é: pela paciência para lidar com as minhas partidas constantes, por me fazer rir com as suas odisseias de electrofisiologia, pelas expressões hilariantes que faz quando eu digo as coisas absurdas que digo, pelos muitos cigarros que lhe cravei e, acima de tudo, pela companhia diária, quer dentro, quer fora do laboratório. És pequenina e resmungona, mas eu gosto de ti assim. Já agora, já que aqui estás, queres ir lá abaixo?

Ao Francisco Mouro, não só por me ter ensinado e ajudado a fazer comportamento, mas também pelas conversas acerca dos testes e do que estamos mesmo a fazer quando testamos animais, e por ter tido a disponibilidade para rever o capitulo de metodologia desta dissertação – este trabalho é melhor pela tua ajuda.

Aos *Xapellis*, mas em especial a três de vós: ao Rui Rodrigues, por ser o melhor público possível e se rir mesmo das minhas piores piadas, e pelas conversas quer sobre canabinóides, quer sobre a vida. À Marta Alonso, por ser uma das pessoas mais doces e simpáticas que já tive o prazer de conhecer, e cujo entusiasmo, boa disposição e sentido de humor melhoram qualquer dia, mesmo quando esse dia é um sábado ou domingo de Agosto passado no laboratório. E à Sara Raquel Paulo, por me ensinar a pipetar (e não me deixar esquecer disso), por ter a paciência e disponibilidade para me ajudar e ensinar a fazer *imunos*, por ainda não me ter batido apesar de eu lhe dar razões mais que suficientes para o fazer, e por – apesar de às vezes não nos entendermos – ser uma das melhores pessoas que conheci nos últimos tempos (tenta não ficar com o ego muito cheio).

À Sandra Vaz pela disponibilidade, paciência, encorajamento que me deu enquanto me ensinava a dissecar, e por me ter ajudado nas dissecções de uma das experiências. Mais ainda, pelo papel crucial que desempenha no laboratório, e por toda a ajuda que nos presta a todos diariamente. E à Joana Gomes, estudante da Sandra, por ser uma das minhas vitimas e ter um excelente sentido de humor acerca disso, pela companhia no tabagismo, e por me deixar lembrar-lhe diariamente que a vida é sofrimento.

À Varél… Valéria Martins e à "Outra" (Beatriz Pereira), por terem sido as minhas *underlings* emprestadas, que tanto me ajudaram a fazer handling e a correr testes de comportamento. Mais que isso, por serem duas miúdas espectaculares, que trouxeram imensa animação, energia e "boa procrastinação" aos meus dias no laboratório, e que – por isso – fizeram dos meses que cá estiveram uma das minhas alturas favoritas. Podem ter radicalizado os meus ratos contra mim, e estar lá longe na Suécia, mas eu perdoo-vos e não me esqueço de vocês.

À Alexandra Maralhas, sem a qual eu ainda estaria à espera do meu primeiro frasco de HU-210, e que tornou – pela sua dedicação e trabalho incansável – este, e outros projectos futuros, possíveis. Mais ainda, um enorme obrigado pelo teu interesse e entusiasmo pelo meu trabalho.

À Alexandra Botelho e à Cristina Varandas, por toda a ajuda que me deram ao longo deste processo, e por me darem conversa quando eu estava a evitar ir fazer alguma coisa que não queria fazer. A vossa dedicação aos alunos e ao vosso trabalho é admirável.

Tenho também de agradecer ao Professor Paulo Ventura, da Faculdade de Psicologia da Universidade de Lisboa, o responsável por eu ter começado a fazer investigação, e com quem partilho a minha mais longa colaboração profissional. Agradeço-lhe não só por isso, mas por ser um modelo de como fazer ciência, de como há sempre mais por fazer e por descobrir, e por me ter dado – e continuar a dar – imensas oportunidades. Mais ainda, devo-lhe um agradecimento por ser no contexto do seu grupo que conheci e criei amizade com todo um conjunto de pessoas fantásticas – o João Delgado, o José Carlos Guerreiro, o Bruno Faustino e o António Fernandes – com os quais é um prazer, um orgulho, e uma honra, trabalhar. Um obrigado a todos vós.

Aos meus dois melhores amigos de longa data, as minhas fofinhas, o André Marques e o Carlos Santos, não há muito que seja necessário dizer. Mas isso nunca me impediu de falar, portanto aqui vai: obrigado pelos mais de 10 anos de amizade, pelas histórias e momentos de riso, por não me mandarem calar quando não falo de mais nada senão de trabalho, e por continuarem a ser as duas pessoas com quem posso falar de tudo e com os quais sei que posso contar seja para o que for. Aconteça o que acontecer, isto é para sempre, e se precisarem de um alibi para alguma eventualidade, sabem a quem ligar.

Não poderia, em boa consciência, deixar também de agradecer aos animais que deram a vida por este trabalho. Espero ter sido um bom dono, e ter feito a vossa curta vida o mais confortável possível. Num

mundo ideal ter-vos-ia trazido a todas para casa comigo, e peço desculpa por não o poder ter feito. E, igualmente, não poderia não agradecer ao animal sem a qual a minha vida seria muito menos feliz: a Nina, o melhor cão que alguém poderia querer. Não há festinhas suficientes para te agradecer, mas eu tento na mesma.

Termino com um obrigado à minha família, mas em especial às duas pessoas sem as quais esta minha aventura não teria sido possível. Ao meu avô Quim e à minha avó Minda, que nunca me deram nada senão amor e aceitação incondicional, e que trago comigo no coração todos os dias. Se, neste último ano, não estive tão presente nas vossas vidas quanto gostaria, foi porque quis que o voto de confiança que fizeram em mim não fosse desperdiçado, e espero – acima de tudo – ter-vos deixado orgulhosos do vosso primeiro neto. Não há um dia em que não me sinta grato pela vossa ajuda, e por vos ter na minha vida.

Palavras, por muitas que sejam, não são o suficiente para agradecer tudo o que tenho para agradecer a todos vós. Resta-me tentar que, não só estas palavras, mas as minhas acções, vos mostrem o quanto gosto de todos vocês, e o quão sortudo me sinto ser por vos ter na minha vida. A todos, sem excepção, o mais profundo e sincero obrigado.

“There is no easy way from the earth to the stars.”

-

Lucius Annaeus Seneca

“The mind adapts and converts to its own purposes the obstacle to our acting. The impediment to action advances action. What stands in the way *becomes* the way.”

-

Marcus Aurelius

Scientific Production

During the year during which the current work was developed I was invited to participate in the writing of several review articles, two of which have since been published:

- **Ferreira, M.F.***, Castanheira, L. Sebastião, A.M., Telles-Correia, D. (2018). Depression assessment in clinical trials and pre-clinical tests: a critical review. *Current Topics in Medicinal Chemistry*, 18(19), 1677-1703
- Castanheira, L., **Ferreira, M.F.***, Sebastião, A.M., Telles-Correia, D. (2018). Anxiety assessment in pre-clinical tests and in clinical trials: a critical review. *Current Topics in Medicinal Chemistry*, 18(19), 1656-1676
- Rodrigues, R.S., Lourenço, D., Paulo S.L., Mateus, J., Moreira, J.B., **Ferreira, M.F.**, Mouro, F.M., Ribeiro, F.F., Sebastião, A.M., Xapelli, S. (in review). Endocannabinoid actions on neural stem cells: implications for physiopathology. *Molecules*.

Furthermore, the scientific content of the present dissertation, has been presented in poster sessions at several national and international conferences, and constitutes the majority of a manuscript currently in preparation for publication:

- **Ferreira M.F.,** Paulo S.L., Fonseca-Gomes J., Rei N., Vaz S.H., Mouro F.M., Sebastião A.M. *Of rats, cannabinoids, and the blues: the unexpected short- and long-term effects of chronic adolescent HU-210 exposure on affective behavior.*

Note: Asterisks (*) denote co-first authorship.

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List of Abbreviations

2-AG – 2-Arachidonoyl-glycerol

5-HT – Serotonin (5-hydroxytryptamine)

5-HT_{1A}R – Serotonin type 1A receptor

5-HT_{2A}R – Serotonin type 2A receptor

AA – Arachidonic acid

ABHD – Alpha/beta-hydrolase domain-containing

AC – Adenylyl cyclase

aCSF – Artificial cerebral spinal fluid

ACTH – Adrenocorticotropic hormone

AEA – Anandamide (N-arachidonoyl-ethanolamine)

AHA1 – Activator of 90 kDa heat shock protein ATPase homolog 1

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor

APAP – Active place avoidance paradigm

APS – Ammonium persulfate

Arc – Activity-regulated cytoskeleton-associated protein

ATP – Adenosine triphosphate

BDNF - Brain derived neurotrophic factor

BrdU – 5-bromo-2'-deoxyuridine

BSA – Bovine serum albumin

Ca^{2+} – Calcium (2+) ion

$CaCL_2$ – Calcium chloride

cAMP – 3',5'-cyclic adenosine monophosphate

CB_1R – Cannabinoid receptor type 1

CB_2R – Cannabinoid receptor type 2

CBRA – Cannabinoid receptor agonist

CMS – Chronic mild stress

CO_2 – Carbon dioxide

CORT – Corticosterone

COX-2 – Ciclooxigenase-2

CPu – Caudate-putamen

CRF – Corticotropin releasing factor

CSF – Cerebral spinal fluid

CUD – Cannabinoid use disorder

CZ – Central zone of the OFT

D_1R – Dopamine receptor type 1

D_2R – Dopamine receptor type 2

DA – Dopamine

DAG – Diacylglycerol

DAT – Dopamine transporter

DG – Dentate gyrus

DGLα – DAG lipase α

DMSO – Dimethyl sulfoxide

DOPAC – L-3,4,-dihydroxyphenylacetic acid

dRN – Dorsal raphe nuclei

DSI/E – Depolarization induced suppression of inhibition/excitation

eCB – Endocannabinoid

eCB-LTD – Endocannabinoid mediated long-term depression

eCB-STD – Endocannabinoid mediated short-term depression

ECS – Endocannabinoid system

EDTA – Ethylenediamine tetraacetic acid

EMT – Endocannabinoid membrane transporter

EPM – Elevated plus maze

ER – Endoplasmatic reticulum

ERK – Extracellular signal-regulated kinase

FAAH – Fatty acid amide hydrolase

FABP – Fatty acid binding protein

FST/mFST – Forced swim test/modified forced swim test

GABA – γ-aminobutyric acid

$GABA_AR$ - GABA A receptor

$GABA_BR$ – GABA B receptor

GAD – Glutamate decarboxylase

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GAT-1 – GABA transporter 1

GIRK – G-protein coupled inwardly rectifying K^+ channels

GluA1 – AMPAR subunit 1

GluA2 – AMPAR subunit 2

GluN2A – NMDAR subunit 2A

GluN2B – NMDAR subunit 2B

GPCR – G-protein coupled receptor

HBT – Holeboard test

HPA axis – Hypothalamic-pituitary-adrenal axis

HSP90 – 90 kDa heat-shock protein

i.p. – Intraperitoneal injection

Iba1 – Ionized calcium-binding adapter molecule 1

IL-10 – Interleukin-10

iNOS – Inducible nitric oxide enzyme

IQ – Intelligence quotient

IQR – Interquartile range

IZ – Intermediate zone of the OFT

JNK – c-Jun N-terminal kinase

K^+ – Potassium ion

KCl – Potassium chloride

KOR – Kappa (κ) opioid receptor

LC – Locus coeruleus

LDBT – Light-dark box test

LFP – Local field potential

LHb – Lateral habenula

LOX – Lipoxygenase

MAGL – Monoacylglycerol lipase

MAPK – Mitogen activated protein kinase

MBT – Marble burying test

mGluR – Metabotropic glutamate receptor

$MgSO_4$ – Magnesium sulfate

MOR – Mu (μ) opioid receptor

mPFC – Medial prefrontal cortex

MSI/E – Metabotropic induced suppression of inhibition/excitation

MTORC1 – Mammalian/mechanistic target of rapamycin complex 1 pathway

MWM – Morris water maze

NA – Noradrenaline

Na_3VO_4 – Sodium orthovanadate

NAAA – N-acylethanolamine hydrolyzing acid amidase

NAc – Nucleus accumbens

NaCl – Sodium chloride

NaF – Sodium fluoride

NaH_2PO_4 – Sodium dihydrogen phosphate

NAPE – N-acylphosphatidylethanolamine

NMDAR – N-methyl-D-aspartate (NMDA) receptor

NO – Nitric oxide

NOPRT – Novel object place recognition test

NORT – Novel object recognition test

NP40 – Nonidet® P40 substitute

NSFT – Novelty suppressed feeding test

O_2 – Molecular oxygen

OCD – Obsessive-compulsive disorder

OFC – Orbitofrontal cortex

OFT – Open field test

pCREB – phosphorylated cAMP response element-binding protein

PFC – Prefrontal cortex

PFPT – Palatable food preference test

PI – Phosphatidylinositol

PI3K – Phosphatidylinositol-3-kinase

PKA/PKB/PKC – Protein kinase A/B/C

PLC – Phospholipase C

PND – Postnatal day

PP – Perforant path

PSA-NCAM – Polysialylated-neural cell adhesion molecule

PSD95 – Post-synaptic density protein 95

PTX – Pertussis toxin

PVDF – Polyvinylidene difluoride

PVN – Paraventricular nucleus of the hypothalamus

PZ – Peripheral zone of the OFT

Q1 – First quartile

Q3 – Third quartile

r – Pearson correlation coefficient

RIPA – Radio immunoprecipitation assay

RT – Room temperature

SC/SCs – Synthetic cannabinoid/s

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – Standard error of mean

SERT – Serotonin transporter

SIT – Social interaction test

SMSNT – Social motivation and social novelty task

SN – Substantia nigra

SPT – Sucrose preference test

SSI – Slow self-Inhibition

SSRI – Selective serotonin reuptake inhibitor

TBS-T – Tris buffered saline with Tween®20

TCA – Tricyclic antidepressant

TEMED – N,N,N',N'-tetramethylethane-1,2-diamine

TH – Tyrosine hydroxylase

THC – Δ9-tetrahydrocannabinol

t-LTD – Spike-timing dependent long-term depression

TNFα – Tumor necrosis factor α

TRPV 1 – Transient receptor potential cation channel subfamily V member 1

VAMP2 – Vesicle-associated membrane protein 2

VEGF – Vascular endothelial growth factor

VEH – Vehicle solution

VGCC – Voltage-gated calcium channel

vGluT1 –Vesicular glutamate transporter 1

vSub – Ventral subiculum

VTA – Ventral Tegmental Area

WT – Wild type

Resumo

Os canabinóides, agonistas dos receptores do sistema endocanabinóide (SEC), são as drogas ilegais mais consumidas no mundo, sendo os adolescentes um dos grupos etários em que o consumo destas substâncias é mais prevalente.

A adolescência representa um período crítico do neurodesenvolvimento, no qual o sistema nervoso central é extensamente reorganizado – sendo, também, um período de vulnerabilidade aumentada aos efeitos de influências externas, como por exemplo drogas de abuso. Criticamente, grande parte destas alterações neuronais são mediadas e/ou moduladas pelo SEC sendo, portanto, expectável que o uso de drogas que interagem com esse sistema cause alterações profundas, e possivelmente persistentes, no funcionamento do sistema nervoso. Congruentemente, dados obtidos quer com humanos, quer com roedores, sugerem que a exposição crónica adolescente a canabinóides tem efeitos deletérios quer ao nível da função neurocognitiva, quer ao nível do funcionamento afectivo.

Assim, estudos epidemiológicos em populações humanas têm demonstrado que indivíduos adultos, que enquanto adolescentes foram consumidores crónicos de canabinóides, apresentam um risco aumentado de serem diagnosticados com perturbações de ansiedade e/ou perturbações depressivas. Mais ainda, devido a um conjunto ainda não totalmente estudado de factores, este aumento de risco é mais marcado na população do sexo feminino.

Em linha com estas observações, estudos experimentais com roedores têm consistentemente demonstrado que a exposição crónica adolescente a canabinóides induz um conjunto profundo e diversificado de alterações a nível molecular, morfológico, estrutural, funcional e comportamental. Em relação ao último, estudos comportamentais têm repetidamente demonstrado que animais adultos expostos a canabinóides durante a adolescência apresentam défices não só em tarefas de função cognitiva, como em testes de função afectiva – apresentando alterações comportamentais que sugerem um efeito prodepressivo desta exposição, que, replicando os dados humanos, é mais marcado em fêmeas.

Uma limitação da literatura até agora é, no entanto, o uso de um conjunto limitado de canabinóides. De facto, praticamente todos os estudos usaram apenas uma de três substâncias, o que levanta a possibilidade de existirem outros canabinóides cujos efeitos diferem dos até agora observados, o que – a ser verdade – representaria um problema importante na literatura.

O presente trabalho pretende averiguar essa possibilidade, ao estudar o HU-210, um potente agonista total e não-selectivo dos receptores canabinóides 1 (CB_1R) e 2 (CB_2R). Apesar de este fármaco ser amplamente usado em investigação sobre o SEC, e já ter sido encontrado em substitutos sintéticos de canábis, não existe – à data – nenhum relato publicado acerca do impacto que a exposição crónica adolescente a HU-210 possa ter no funcionamento afectivo. Assim, o presente trabalho consiste num conjunto de quatro experiências desenhadas para caracterizar esses efeitos.

Na primeira experiência ratos, Wistar fêmea com 35 dias de idade (PND 35) foram administrados HU-210 diariamente, durante 15 dias, num plano de doses ascendentes (PND 35-39: 25µg/kg; PND 42-46: 50µg/kg; PND 49-53: 100µg/kg, ou solução veiculo equivalente). Após o fim da administração, os animais foram deixados durante 27 dias – de modo a que efeitos residuais, ou resultantes de abstinência, pudessem ser minimizados e permitindo que os animais atingissem a idade adulta – ao fim dos quais foram testados numa bateria de testes comportamentais. Especificamente, para medir alterações ao nível do comportamento ansioso, os animais foram testados no *Elevated Plus Maze* (EPM; PND 80), *Open Field Test* (OFT; PND 80 e 81) e *Marble Burying Test* (MBT; PND 91). Para determinar os efeitos do tratamento no comportamento social foi utilizado o *Social Interaction Test* (SIT; PND 82). Finalmente, para avaliar os efeitos da exposição a HU-210 nas dimensões de *stress-coping* e de resposta à recompensa, do comportamento depressivo, os animais foram testados no *Modified Forced Swim Test* (mFST; PND 85) e no *Sucrose Preference Test* (SPT; PND 88-91), respectivamente. Adicionalmente, o peso dos animais foi registado ao longo da duração da experiência.

Análise dos resultados obtidos revelou que, tal como descrito previamente para outros canabinóides, a exposição a HU-210 não induziu alterações persistentes ao nível do comportamento ansioso, em nenhum dos três testes. Contrariamente ao anteriormente descrito, não foi observada qualquer alteração no SIT, indicando a ausência de efeitos persistentes. No que diz respeito ao comportamento depressivo, foi registado um decréscimo ligeiro no comportamento de trepar, no mFST – sugerindo a possibilidade de alterações de *stress-coping* – sem qualquer diferença nos outros comportamentos. No SPT não foram encontradas diferenças quer na quantidade de sacarose consumida, quer na preferência relativa por sacarose, indicando que a resposta à recompensa não está alterada. Finalmente, em linha com estudos anteriores, a exposição a HU-210 induziu decréscimos marcados no ganho de peso, que persistiram durante

15 dias após o fim da administração.

Dado que os resultados da experiência 1 não foram os esperados, e que várias limitações foram identificadas no protocolo, a experiência 2 foi desenhada para – de novo – testar os efeitos a longo-termo da exposição crónica adolescente a HU-210. Assim, ratos Sprague-Dawley fêmea receberam duas injecções diárias de HU-210 durante 11 dias, seguindo um padrão de doses ascendentes (PND 35-37: 25µg/kg; PND 38-41: 50µg/kg; PND 42-45: 100µg/kg ou veículo equivalente). Após o término da administração, os animais foram deixados em repouso durante 30 dias, ao fim dos quais lhes foi aplicada a mesma bateria de testes comportamentais usada na experiência anterior. Adicionalmente, de modo a determinar os efeitos da exposição adolescente nos níveis de proteína CB_1R, amostras de tecido do hipocampo, estriado e córtex pré-frontal, foram recolhidas após o fim da bateria comportamental (PND 88), tendo os níveis de CB_1R sido avaliados através de *western blotting*.

Tal como na experiência 1, não foram encontradas quaisquer alterações no comportamento ansioso, ou no comportamento social, e foi observado um decréscimo marcado no ganho de peso que – mais uma vez – persistiu durante 15 dias pós-última administração. No entanto, em contraste quer com a experiência anterior, quer com a literatura, no mFST também não foram observadas alterações. Semelhantemente, no SPT, o desempenho foi igual entre grupos. Em linha com a ausência de alterações comportamentais, não se detectaram alterações nos níveis de proteína CB_1R em nenhuma das três regiões estudadas.

Dada a discordância entre os resultados obtidos na experiência 2 e o descrito na literatura, a experiência 3 consistiu em avaliar se a administração adolescente de HU-210 tinha de facto algum efeito mensurável a curto-prazo que poderia ter desaparecido, durante o interregno de 30 dias entre a última administração e o início dos testes. Para isso uma nova série de ratos Sprague-Dawley fêmea foi manipulada como descrito na experiência 2, e testada no OFT e mFST, nos dois dias após a última administração de HU-210 (PND 46-47). Adicionalmente, amostras de tecido para *western blot* foram recolhidas no dia após o fim dos testes comportamentais (PND 48).

Surpreendentemente, apesar de nenhuma alteração ter sido encontrada no OFT, no mFST os animais mostraram um padrão comportamental marcado e sugestivo de um efeito antidepressivo do tratamento, com decréscimos no tempo passado em imobilidade e aumentos no tempo passado a trepar. Mais ainda, análise do *western blot* revelou um decréscimo de cerca de 50% nos níveis de proteína CB_1R, na região

hipocampal, sem alterações nas restantes.

Uma vez que os resultados na experiência 3, no que respeita aos parâmetros do mFST, foram inteiramente inesperados, e as alterações moleculares encontradas foram incongruentes com um efeito antidepressivo, a experiência 4 foi desenhada para mais uma vez avaliar os efeitos imediatos da exposição adolescente a HU-210, recorrendo a outros dois testes, frequentemente usados para testar ansiedade e depressão, o EPM e o SPT, respectivamente. Assim, uma nova série de ratos Sprague-Dawley fêmea foi manipulada como descrito nas experiências 2 e 3, e testada no EPM (PND 46) e SPT (PND 46-49) nos dias que se seguiram à última administração de HU-210.

Em linha com o encontrado na experiência 3, não foram observadas alterações no comportamento ansioso no EPM. No entanto, em contraste marcado, no SPT, o grupo tratado com HU-210 consumiu significativamente menos sacarose que os controlos e mostrou, igualmente, um decréscimo acentuado na preferência pela mesma – alterações sugestivas de um efeito prodepressivo.

No geral os resultados obtidos ao longo das quatro experiências sugerem que, apesar de o HU-210 ser capaz de induzir alterações marcadas no funcionamento afectivo, estas alterações desaparecem após algum tempo. Mais ainda, o facto deste fármaco não induzir efeitos a longo termo sugere a possibilidade de que diferenças nas características farmacológicas dos vários canabinóides possam ter influência importante e imprevisível, nos resultados observados na literatura. Especificamente, é possível que a ausência de efeitos duradouros após exposição crónica adolescente a HU-210, derive de diferenças farmacodinâmicas deste canabinóide quer ao nível da sua interacção com o SEC, quer ao nível de possíveis interacções com outros sistemas de neurotransmissão/neuromodulação. Assim, a principal conclusão derivada deste trabalho prende-se com a noção de que, ao usar apenas um conjunto limitado de agonistas dos receptores canabinóides, se está a incorrer dois riscos: por um lado, o risco de ignorar a totalidade dos possíveis efeitos da modulação do SEC, e por outro, se formarem conclusões extrapoladas a partir de dados obtidos com vários fármacos diferentes, cuja farmacologia e efeitos podem não ser totalmente comparáveis. Ambos estes riscos têm fortes implicações para a interpretabilidade e utilidade da investigação feita usando canabinóides e acerca do potencial benéfico e/ou deletério da manipulação farmacológica do SEC.

Palavras Chave: canabinóides, adolescência, HU-210, depressão, ansiedade.

Abstract

Cannabinoids, drugs acting as agonists at the cannabinoid receptors comprising the endocannabinoid system (ECS), are the most widely used illegal drug class in the world, with adolescents being one of the age group where the use of such drugs is most prevalent. Adolescence represents a critical neurodevelopmental period, during which the central nervous system undergoes extensive reorganization, with this development being heavily mediated by the ECS. Thus, it is expectable that the adolescent use of drugs targeting that system will lead to profound, and possibly permanent, alterations in nervous system functioning. Accordingly, both human and rodent studies suggests that chronic adolescent exposure to cannabinoids induces deleterious effects at both the cognitive and affective functioning levels:

Epidemiological studies of human populations have shown adults, who were chronic cannabinoid users as adolescents, to be at an increased risk of being diagnosed with both anxiety and/or depressive disorders, with risk being even greater for females. Similarly, rodent experimental studies have consistently demonstrated that chronic adolescent cannabinoid exposure leads to lasting deficits not just in tasks of cognitive function, but in tests of affective functioning, as well – with behavioral alterations suggesting a prodepressant-like effect of cannabinoid treatment which, as in humans, is more marked in females.

One limitation of the literature is, however, the overreliance on a limited set of cannabinoids, raising the possibility that other, yet unstudied cannabinoids, may have differing effects from those reported thus far – a significant problem for the field, if true. As such the present work aimed, through four experiments, to test that possibility, by characterizing the affective impact of chronic adolescent exposure to HU-210 – a potent non-selective full-agonist at both cannabinoid receptors 1 (CB_1R) and 2 (CB_2R) – that, despite being widely used in ECS research, has yet to be studied in this regard.

In the first experiment, female Wistar rats aged 35 days (PND 35) were administered HU-210 daily, for a 15-day, in an escalating dosing schedule (PND 35-39: 25µg/kg; PND 42-46: 50µg/kg; PND 49-53: 100µg/kg, or equivalent vehicle solution). Following a 27-day washout period animals were put through a battery of behavioral tests: to assess anxiety-like behavior the Elevated Plus Maze (EPM; PND 80), Open Field Test (OFT; PND 80-81) and Marble Burying Test (MBT; PND 91) were used; to assess social behavior, the Social Interaction Test (SIT; PND 82) was employed; to assess the stress-coping and reward

functioning dimensions of depressive-like behavior the Modified Forced Swim (mFST; PND 85) and the Sucrose Preference Tests (SPT; PND 88-91) were used, respectively.

Data showed that, as previously described for other cannabinoids, adolescent exposure to HU-210 did not lead to persistent alterations at the level of anxiety-like behavior. However, contrarily to what had been previously described, in the SIT no alteration was observed, suggesting no lasting treatment-induced impairments. With regards to depressive-like behavior, a slight decrease in climbing behavior was observed in the mFST – suggesting the possibility of altered stress-coping – but no changes were found in any of the other behaviors scored. Moreover, no changes were found in the SPT, pointing towards reward functioning being intact.

Experiment 2 was designed to again test the long-term effects of chronic adolescent HU-210 exposure, controlling for confounds that may have biased the results of experiment 1. As such, female Sprague Dawley rats, received twice-daily intraperitoneal injections of HU-210 for a period 11-days, following an escalating dosing schedule (PND 35-37: 25μg/kg; PND 38-41: 50μg/kg; PND 42-45: 100μg/kg or equivalent vehicle solution). After a 30-day washout period, animals were tested using the same behavioral testing battery used in the previous experiment. Additionally, so as to determine the effects of exposure on CB_1R protein levels, through western blotting, tissue samples were collected from the hippocampus, striatum and prefrontal cortex (PND 88).

As was the case in experiment 1, no changes were found in anxiety-like or social behaviors. However, in contrast with both the previous experiment and the literature, no changes were observed in any of the mFST parameters, nor in the SPT. Furthermore, in line with the absence of behavioral alterations, CB_1R protein levels were found to be unaltered in all the three brain regions studied.

Given the discrepancy between the results obtained in experiment 2 and those described in the literature, experiment 3 was performed so as to determine whether adolescent HU-210 administration did, in fact, have any measurable short-term effect, that might be normalized during washout. To that end a new set of female Sprague-Dawley rats, was manipulated as described in experiment 2, and tested in both the OFT and the mFST, in the two days following the last drug injection (PND 46-47). Additionally, tissue samples for western blotting were collected from the same brain regions (PND 48).

Surprisingly, in the mFST animals presented a behavioral pattern suggestive of an antidepressant-like effect of treatment – with decreased in the time spent in immobility and increased time spent climbing. Critically, this effect occurred in the absence of any alterations in the OFT. Moreover, a decrease of approximately 50% in hippocampal CB_1R protein levels was observed, with no changes in the remaining regions studied.

Since the results of experiment 3 were unexpected, and the molecular alterations observed were incongruous with an antidepressant-like effect, experiment 4 was performed to complement them, by using two other tests: the EPM and the SPT. As such, a new set of female Sprague-Dawley rats was manipulated as described in experiments 2 and 3, and tested in the EPM (PND 46) and SPT (PND 46-49) on the days following the last drug administration.

In line with the previous experiment, no alterations were observed in anxiety-like behavior. However, contrastingly, the HU-210-treated group presented markedly decreased sucrose intake and relative sucrose preference, in the SPT – suggesting a prodepressant treatment effect.

In general, the results obtained across the four experiments suggest that, despite HU-210 being able to alter affective functioning, these alterations are normalized after sufficient washout time. Moreover, the fact that this drug did not induce long-term effects suggests the possibility that differences in the pharmacological properties of cannabinoids – either in terms of ECS or non-ECS interactions – may influence results observed in the literature, in important and unpredictable ways. As such, the primary conclusion derived from the present work pertains to the notion that, by relying on a limited set of cannabinoid receptor agonists, one may be at risk of ignoring the totality of the possible effects of ECS modulation and of forming possibly erroneous conclusions, extrapolated from data obtained with different drugs, whose pharmacology and effects may not be totally comparable. Both of these risks have strong implications for the interpretability and usefulness of cannabinoid research, and into the beneficial and/or deleterious potential of pharmacological manipulation of the ECS.

Keywords: cannabinoids, adolescence, HU-210, depression, anxiety.

Chapter 1 – Introduction

1 – Cannabinoid Use and Abuse Worldwide

Cannabis sativa (along with its many derivative preparations, such as hashish) is the most widely consumed illegal drug in the world, having been used at least once in the last 12 months by an estimated 2.7-4.9% of the global population (183±55 million people)[1], and is used daily by an estimated 1% of the European population[2]. Indeed, the use of this substance is so prevalent that it is only surpassed by the, more widely available, legal psychoactive substances, such as alcohol, tobacco, and caffeine[1]. Critically, a large segment of cannabis consumers are adolescents: the 2015 European School Survey Project on Alcohol and Other Drugs, found that, in a sample of 96046 students aged 15-16, from 35 European nations, 16% reported having used cannabis at least once in their lives, 7% reported using it in the last 30 days, and 3% reported having first used cannabis at ≤ 13 years of age[3]. These figures, which represent increases from previous years[1,3], are likely to grow in the future, given the increasing support for the decriminalization/legalization of cannabis across the American and European continents[4], and the simultaneous decrease in the perceived harmfulness of cannabis use amongst teenagers[5].

Concomitantly with the increase in cannabis use in recent years, there have been reports, starting in 2008, of the use of synthetic cannabinoids (SCs)[6], as legal alternatives to cannabis. Indeed, between 2008 and 2016 more than 240 different new cannabinoid compounds, spanning multiple chemical classes, were identified in commercially available products[1]. These products – sold in highly branded packaging, under the guise of being incense or potpourri, and "not for human consumption", in an attempt to skirt drug laws[6] – are often attractive to adolescents due to their perceived legality, as well as their lower cost and greater ease of access[7,8], relative to cannabis. However, despite this, there is a dearth of global epidemiological data regarding, the prevalence and patterns of SC adolescent use: a recent study found that 3.5% of American high school seniors reported having used SCs in the past year[9], whereas studies done in Spain, Sweden and Germany have estimated adolescent use of SCs to have a prevalence of 0.8%, 3.2% and 6%, respectively[6].

Despite being sold as legal alternatives to it, SCs possess characteristics that make them markedly different from and, indeed, likely more dangerous than cannabis. Unlike Δ9-tetrahydrocannabinol (THC),

the main psychoactive compound in cannabis – which is responsible for the majority of both its pleasant (e.g., euphoria, increased appetite, heightened sense perception, relaxation and pain reduction) and unpleasant effects (e.g., short-term memory deficits, xerostomia, increased anxiety and motor impairment), by acting as a partial agonist at the human cannabinoid receptor type 1 (CB_1R)[10] – the overwhelming majority of SCs are high potency, high affinity, full agonists at this receptor. Furthermore, given the well-established biphasic dose-effect relationship that characterizes THC – whereby at low doses users report mainly pleasant effects, whereas unpleasant effects become more prevalent at higher doses[11] – these pharmacological differences are likely to underlie the more severe effects and adverse psychological reactions reported by SC users, such as extreme anxiety, hallucinatory phenomena, as well as psychotic and suicidal episodes[12]. Moreover, there is increasing evidence that SCs may also be more physically toxic than cannabis, with reports detailing cases of hyperemesis, hyperthermia, cardiovascular problems, acute kidney injury, seizures, and loss of consciousness, following SC use, which have in some cases led to fatalities[12]. Importantly, at least a few of these physical symptoms (e.g., seizures[13]) have been directly tied to activity of SCs at the CB_1R.

Given both the increases in access to, and use of, both cannabis (especially higher potency strains/preparations[14–16]) and SCs, it is unsurprising that concomitant increases in the prevalence of, and search of treatment for, cannabinoid use disorders (CUD) have been reported. Importantly, not only do the majority of treatment entrants report having begun cannabis use during adolescence[17,18], but there has also been a steady decrease in the age at which individuals first seek treatment for these disorders[1]. Moreover, despite increasing access to treatment[19], it is estimated that only 15-37% of individuals treated for CUD will maintain abstinence[20], with one of the main reasons for this being the manifestations of cannabinoid withdrawal syndrome[21]. This well characterized syndrome presents mostly in heavy chronic users, and consists of muscle weakness, restlessness, sweating, dysphoria, insomnia, anxiety and craving[22]. Furthermore, as is the case with their acute effects, this syndrome seems to be much more pronounced in consumers of SCs[23,24], often requiring hospitalization[25], and is reported to begin as early as 15 minutes after the last use[24].

Given this picture of widespread cannabinoid use and abuse, it is quite interesting that there is also a parallel, and increasingly higher, interest in the medicinal use of cannabinoid-based therapeutics[26]. Indeed,

cannabinoids and other drugs targeting the endogenous cannabinoid system (endocannabinoid system; ECS) are being investigated as possible therapeutics for numerous conditions such as chronic and neuropathic pain[27,28], chemotherapy induced nausea and vomiting[29,30], obesity[31–33], AIDS and cancer induced cachexia/anorexia[34–36], glaucoma[37], cancer[38,39], epilepsy[40], neurodegenerative diseases such as Huntington's disease and multiple sclerosis[41–45] and neuropsychiatric diseases such as post-traumatic stress disorder[46–48], schizophrenia[49,50], anxiety disorders[51,52] and depressive disorders[53]. However, despite being a highly promising field for experimental therapeutics, development and introduction of new drugs targeting the ECS has been hindered by two main concerns: the psychoactive "on target" side-effects of many of the experimental substances tested[54], and the concerns regarding the consequences of long-term use of these drugs, especially in more vulnerable populations such as children and adolescents[55,56].

As such, a better understanding of the consequences of, and mechanisms behind, the consequences of prolonged treatment with cannabinoids – whether recreational or therapeutic – is key to facilitate the development of strategies to mitigate or revert any lasting effect that this type of exposure may entail, and, thus, both unlock the full therapeutic potential of cannabinoid-based therapeutics, as well as lift the increasing burden imposed on national health systems[57].

2 – The Endocannabinoid System

Cannabinoids exert their actions through interactions with the ECS. This highly conserved neuromodulatory system[58] is known to be expressed at very early stages of embryonic development, being involved in the specification and development of neuronal tissue[59]. Moreover, the ECS has been found to be critically involved in numerous relevant physiological processes, such as neurogenesis[60], the shaping of neuronal connectivity[61], neuroplasticity and the regulation of synaptic activity (see section 2.2), thus explaining the its involvement in processes such as memory and learning[62,63], pain perception[64,65], stress responses[66–68], motor control[69,70], homeostatic regulation[71–73], reproductive functioning[71], reward processing[68,74] and, critically for the present work, affective functioning[75,76].

To more deeply understand how the ECS is involved in this last process (section 2.3) and how the chronic use of cannabinoids during adolescence may impact affective functioning (section 3), the ECS must first be described.

2.1 – Endocannabinoid System Structure

The extensive and diverse processes in which the ECS is known to be involved, derive from the widespread distribution of its constituting elements. Thus, the ECS is comprised of endogenous ligands (endocannabinoids, eCBs), the enzymes responsible for the synthesis and degradation of those ligands, protein transporters, and cannabinoid receptors (fig. 1.1).

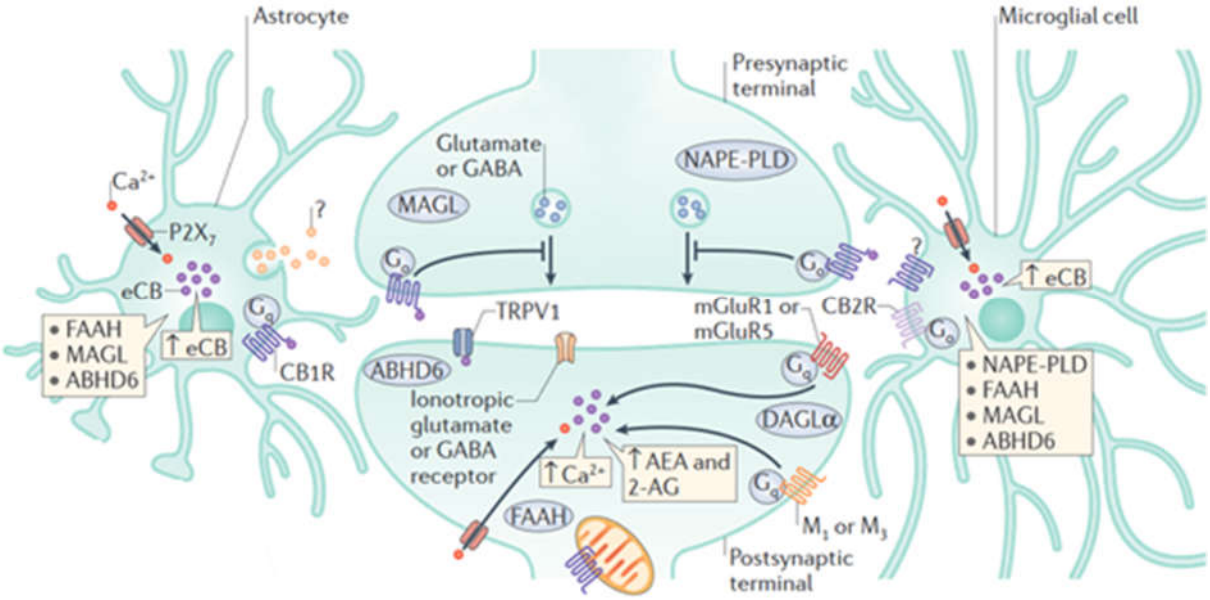

Fig 1.1 – Schematic representation of the endocannabinoid system. The ECS is comprised of receptors, eCBs, the enzymes responsible for the synthesis and inactivation of these ligands, and of the EMT (not pictured). The CB_1R is primarily located on the presynaptic membrane, being activated by AEA and 2-AG released from the postsynaptic neuron. Synthesis occurs in an "on demand" fashion, in response to a number of stimuli, such as the activation of metabotropic glutamate receptors, 2-AG being primarily synthetized postsynaptically, by the DAGLα pathway. AEA synthesis also occurs at the presynaptic neuron, by activation of the NAPE-PLD pathway, and can be released to activate postsynaptic TRPV1R. Fittingly, the primary degradative enzyme for 2-AG, MAGL, is predominantly located in the presynaptic neuron, whereas FAAH – primarily responsible for AEA inactivation – is located postsynaptically. In addition, a number of other enzymes are known to have a role in 2-AG inactivation, such as ABHD6. Moreover, the ECS is also expressed in both astrocytical and microglial cells. In the former, CB_1R couples to a different G-protein than in neurons (G_q and $G_{i/o}$, respectively), and is thought to mediate the release of gliotransmitters, In the former, CB_2R and possibly CB_1R are involved in immune responses mediated by these cells. Figure taken and adapted from Lutz et al.[66]

2.1.1 – Endocannabinoid Synthesis and Release

There are at least seven recognized eCBs[77], of which the most studied are N-arachidonoyl-ethanolamine (anandamide, AEA; fig. 1.2a)[78] and 2-Arachidonoyl-glycerol (2-AG; fig. 1.2b)[79]. Unlike most neurotransmitters and neuromodulators, which are synthetized and stored in vesicles for posterior use, eCBs are primarily synthetized in the postsynaptic neuron, in an "on demand" fashion[a], in response to depolarization-induced calcium (Ca^{2+}) increases and/or the activation of $G_{q/11}$ coupled receptors[77].

[a] It should be noted that, in recent years, evidence has emerged that there may non-on demand production of eCBS, whereby these neuromodulators are stored, and released only when necessary[80].

Anandamide, which acts as a partial agonist at both the CB_1R and CB_2R[10], is synthetized by several pathways[81], the most well characterized of which is the canonical pathway – in which membrane-lipid derived phosphatidylethanolamine is transacylated by an N-acyltransferase, to form N-acylphosphatidylethanolamine (NAPE), which is then hydrolyzed to AEA by the Ca^{2+}-sensitive phospholipase D (NAPE-PLD)[81].

2-AG, which, unlike AEA, is a full agonist at both the CB_1R and CB_2R[82], is synthetized by several different pathways, the most widely studied of which is the PLCβ-DAGα pathway. This pathways seems to be triggered by $G_{q/11}$ activation, leading to phosphatidylinositol (PI) being hydrolyzed by phospholipase C β (PLCβ) to form diacylglycerol (DAG), which is then hydrolyzed by DAG lipase α (DGLα) to form 2-AG[81,83]. Interestingly there is evidence to support the idea that different 2-AG synthesis pathways (some which are PLCβ-independent), started by different stimuli, have distinct physiological roles[84,85]. Once synthetized, these endogenous compounds diffuse across the cellular membrane and into the synaptic cleft where they activate cannabinoid receptors.

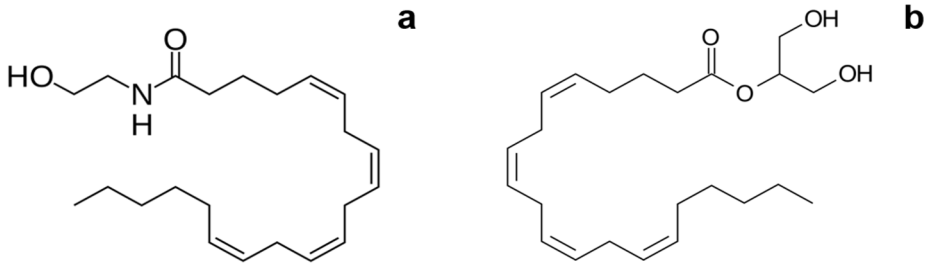

Fig 1.2 - Molecular structures of anandamide and 2-arachidonoyl-glycerol. AEA **(a)** and 2-AG **(b)** are the primary eCBs present in the brain, being responsible for the majority of ECS actions. While AEA acts as a partial agonist at both the CB1R and CB2R, 2-AG is a full agonist at both receptors.

2.1.2 – Cannabinoid Receptors

All known cannabinoid receptors belong to the A-class of the G-protein coupled receptor (GPCR) superfamily[86], with two – the CB_1R and CB_2R – having been amply characterized. In addition, there are several other previously orphan GPCRs (e.g., GPR18, GPR55, and GPR119), whose belonging to the ECS is still disputed – with the most prominent of these being the GPR55[86]. Moreover, in recent years the transient receptor potential cation channel subfamily V member 1 (TRPV1), has also been recognized to be an important element in the ECS[77].

2.1.2.1 – Cannabinoid Receptor Type 1 (CB_1R)

The CB_1R is one of the most abundant receptors in the human central nervous system[87] and is also found in peripheral nervous system[87], as well as in non-nervous tissue such as the liver and adipose tissue[88]. In the brain it is found in high levels in the inner layers of the hippocampus and the olfactory bulb, in the striatum, and in the molecular layer of the cerebellum, with intermediate levels having been found in the frontal, parietal and cingulate cortexes, the amygdala, the hypothalamus, and in some brainstem nuclei[77,87]. At the cellular level, this receptor is predominantly located on the presynaptic terminals of both γ-aminobutyric acid (GABA) releasing neurons (GABAergic neurons), and, to a lesser extent, in glutamatergic neurons[77], where it modulates presynaptic activity. In addition, it also expressed in the postsynaptic membrane – where it has been shown to work as an auto-receptor[89,90] – as well as in astrocytes[91,92], microglia[93] and oligodendrocytes[94].

The CB_1R is typically coupled to $G_{i/o}$ protein, whereby its activation results in the inhibition of adenylyl cyclase (AC) and, therefore, in a decrease of 3',5'-cyclic adenosine monophosphate (cAMP) accumulation[95]. However, it has been shown that this effect is dependent on the specific isoform of AC being expressed, with the opposite effect (i.e., stimulation of AC and cAMP accumulation) occurring in cells expressing AC isoforms 2, 4 and 7, likely through the action of the dissociated $G_{\beta\gamma}$ heterodimer[96]. Moreover, several studies have found that, in some conditions, CB_1Rs are capable of signaling through other G-proteins: CB_1R activation has been demonstrated to lead to increases in AC activity, through G_s protein, in cells where $G_{i/o}$ activation is limited – such as in cells previously treated with pertussis toxin (PTX)[97], or in which other $G_{i/o}$ coupled receptors, such as the dopamine (DA) receptor type 2 (D_2R), are simultaneously activated[98]. Furthermore, some reports have found that CB_1R are capable of signaling through G_q protein, leading to intracellular Ca^{2+} increases[99], most notably doing so in hippocampal astrocytes[92].

$G_{i/o}$ protein activation is also the key component in CB_1R-mediated modulation of ion channels. Indeed, CB_1R activation is known to both increase potassium (K^+) conductance, via activation of A-type and G-protein coupled inwardly rectifying K^+ channels (GIRK)[88,100], and to decrease Ca^{2+} conductance, via inhibition of L-, N- and P/Q-type voltage gated Ca^{2+} channels (VGCC) – through $G_{\beta\gamma}$ mediated interactions[88,100].

Furthermore, in addition to modulating the AC-cAMP pathway and ion channel activity, CB_1R activation also leads to the stimulation of several mitogen activated protein kinase (MAPK) family kinases: depending on the cell type, CB_1R activation has been demonstrated to lead to stimulation of extracellular signal-regulated kinase 1/2 (ERK; alternatively named p42/44 MAPK)[88], through $G_{i/o}$ protein activation, phosphatidylinositol-3-kinase (PI3K) activity via protein kinase B (PKB, also known as Akt kinase)[101], inhibition of AC and protein kinase A (PKA)[102], vascular endothelial growth factor (VEGF) receptor transactivation[103], Src tyrosine kinase FYN activation[104], and activation of Raf MAP kinase through the synthesis of the lipid second messenger ceramide[105]. Finally, CB_1R activation, has also been shown to lead to increased activity of both p38 MAPK and c-Jun N-terminal kinase (JNK), in a cell-type dependent manner[88,100].

2.1.2.2 – Cannabinoid Receptor Type 2 (CB_2R)

The CB_2R was, for a long time, thought to be a peripheral cannabinoid receptor, with no significant expression in the nervous system[106]. Indeed, this receptor is found in high levels in peripheral and immune tissues, such as the spleen, bone, as well as in the gastrointestinal and reproductive systems[106]. However, in the last 15 years, reports have increasingly – but not without significant controversy[106,107] – found CB_2R expression in the nervous system, albeit in much smaller numbers, in comparison with CB_1R. The highest levels of CB_2R have been reported in pyramidal neurons of layers III and V of the orbital, visual, auditory, motor and piriform cortexes, in pyramidal neurons of the CA2 and CA3 regions of the hippocampus, in the striatum, amygdala, and in Purkinje and granular cells of the cerebellum, and more moderate expression levels are found in several brainstem nuclei, such as the substantia nigra pars reticulata and periaqueductal gray[108,109]. At the synaptic level, this receptor, unlike the CB_1R, is found primarily in the postsynaptic membrane[110], and has been demonstrated to act as an auto-receptor via 2-AG signaling, in the CA2 and CA3 regions of the hippocampus[111]. Moreover, CB_2R is also expressed in both microglia and astrocytes, in an activation state-dependent manner[106,112,113] .

CB_2R activation is tied to many of the same intracellular cascades as CB_1R activation: by coupling to $G_{i/o}$ protein, CB_2R activation leads to inhibition of the cAMP-PKA pathway[86,95], and to both increased K^+ conductance and decreased Ca^{2+} conductance, through $G_{\beta\gamma}$ interactions with GIRK[114] and VGCCs[115], respectively. Additionally, CB_2R activation leads to stimulation of the Raf-MAPK cascade, leading to

increased ERK1/2 activity, in four possible ways: through $G_{i/o}$-dependent activation of PLC[116], through decreased PKA activity[117], through stimulation of the PI3K/PKB pathway[94], and through the synthesis of ceramide[118]. Furthermore, CB_2R mediated activation of both the p38 MAPK[119,120] and JNK[120] pathways has also been reported. Finally, CB_2R activation has been shown to lead to an increase in intracellular Ca^{2+} concentration, through PLC mediated Ca^{2+} release from IP_3 controlled calcium stores[121].

However, two important distinctions have to be made between these two receptors: first, unlike CB_1R, CB_2R has not yet been shown to be capable of coupling to G proteins other than $G_{i/o}$, and, secondly, the inhibitory effect that CB_2R activation has over AC is strongly modulated by both expression levels and cell environment, such that in some cells CB_2R activation leads to little or no inhibition of AC activity, whereas in others it fully inhibits it[117].

2.1.3 – Endocannabinoid Uptake

Once eCBs have activated the cannabinoid receptors, they are removed from the synaptic cleft so as to be intracellularly degraded[81]. However, the mechanism through which eCB uptake occurs is highly debated, and not yet fully elucidated[122,123]. Indeed, there is a long standing idea of an eCB membrane transporter (EMT)[124], which posits the existence of a (yet to be characterized) membrane transporter which would transport eCBs (especially AEA) to the intracellular space[122,123]. This transporter is purported to be saturable, to work in a time and temperature-dependent manner, and to be capable of being selectively inhibited[123]. Accordingly, drugs have been developed that greatly decrease the rate at which AEA is removed from the extracellular space[125]. Moreover, experiments have demonstrated AEA uptake to be ATP- and ion gradient-independent, thus excluding the possibility of EMT working like other transporters (such as the DA transporter)[125–127]. However, several aspects of this hypothesis have been questioned, giving rise to alternative models:

Based on the findings that the original drugs used to inhibit the putative EMT have shown to also inhibit the activity of the AEA degradative enzyme fatty acid amide hydrolase (FAAH)[128], that selective inhibition of FAAH greatly reduces AEA uptake[129], and that inhibition of EMT does not, on a short time scale (<40s), inhibit AEA uptake[130,131], a passive diffusion model was proposed[130]. In this model it is suggested that it is FAAH – by creating a concentration gradient – that drives AEA uptake, such that AEA passively diffuses across the cellular membrane, and is hydrolyzed intracellularly[130,131]. However, this model has been put

into question, due to the fact that AEA uptake still occurs (albeit in a reduced manner) in cells derived from FAAH$^{-/-}$ knockout mice[132,133], and that newer, more selective, inhibitors of EMT, that do not inhibit FAHH, have shown to still be effective in blocking AEA uptake[132].

Another proposed model for how AEA is uptaken proposes that, instead of entering the cell through the EMT or passive diffusion, AEA might instead do so via endocytosis[134–136]. Specifically, it is suggested that AEA binds to proteins in cholesterol rich domains of the cell membrane (lipid rafts), undergoes rapid endocytosis, and is then transported in vesicles, to be hydrolyzed by FAAH[134–136]. This model is supported by the fact that depletion of cholesterol reduces AEA uptake[135], whereas increased cholesterol augments it[137], that exogenously administered AEA and 2-AG were shown to congregate towards lipid rafts[138], and that AEA has been shown to be accumulated intracellularly in adiposomes[139]. Despite the fact that this model has yet to be fully refuted, it cannot explain why FAAH inhibitors also reduce AEA efflux[140], given it only posits a mechanism for uptake.

A third model posits that AEA is indeed uptaken via passive diffusion, but that, once inside the cell, AEA binds to carrier proteins which take it to be enzymatically degraded[141,142]. Of these carrier proteins the most widely studied are fatty acid binding proteins 5 and 7 (FABP5/7), as well as albumin and heat-shock protein 70, which have already been demonstrated to transport eCBs intracellularly[143,144]. Supporting this model, there are reports that FABP overexpression increased both AEA uptake and hydrolysis, whereas FABP inhibition had the opposite effects[143], and that FABP5$^{-/-}$ knockout mice had a 50% increase in whole-brain AEA levels[145]. However, contrary to this model, there is conflicting data regarding the levels of FABP expression in adult neurons[146], and there is yet no concluding data that excludes the possibility of the existence of an EMT. Additionally, much like is the case with the endocytosis model[134–136], this model cannot explain the inhibitory effects of AEA uptake inhibitors on AEA efflux[140].

Finally, a more recent model, combines the previous model with the idea of the EMT, by suggesting that AEA does indeed bind to a membrane located transporter, which facilitates its diffusion to the cytoplasm[123]. Once in the intracellular space, AEA is suggested to bind to carrier proteins, which then take it to be degraded by FAAH[123]. By suggesting the existence of an EMT, this model allows for the inhibitory effects of AEA uptake inhibitors on AEA efflux.

It should be noted that comparatively little research as yet been done on the mechanism by which 2-AG is removed from the extracellular space, although there is some suggestive evidence that this eCB might be uptaken by the same EMT as AEA, as shown by the fact that 2-AG inhibits AEA uptake[147] and vice versa[148], and that AEA uptake inhibitors also inhibit the uptake of 2-AG[140].

2.1.4 – Endocannabinoid Inactivation

Once eCBs have been removed from the synaptic cleft they are then targeted by several degradative enzymes, which inactivate them. There are distinct enzymatic pathways for AEA and 2-AG metabolism[81]. However, it should be noted that, in addition to these eCB specific pathways, other enzymes, such as ciclooxigenase-2 (COX-2), lipoxygenases (LOXs) and cytochrome P450, have been demonstrated to interact with both 2-AG and AEA, leading to their metabolism or modification into other bioactive compounds. These non-specific pathways will not be covered in the present work, but have been extensively reviewed by Rouzer & Marnett[149].

AEA is hydrolyzed to arachidonic acid (AA) and ethanolamine by one of three enzymes, with complementary distribution. AEA hydrolysis in the central nervous system is mainly mediated by the previously mentioned FAAH[81], as shown by the fact that $FAAH^{-/-}$ knock out mice had greatly increased levels of AEA in the brain[150]. This membrane bound enzyme is located in the endoplasmatic reticulum (ER) of postsynaptic neurons[151]. Despite the shared name, FAAH-2 – the second enzyme responsible for AEA inactivation – has little in common with FAAH, underlining the reduced homology between them[152]: it is located not on the ER, but on adiposomes[153], it is known to be less efficacious in hydrolyzing AEA than FAAH[153], its expression is greater in peripheral tissues[152], such as the liver, and is found in humans but not rodents[152]. Finally, N-acylethanolamine hydrolyzing acid amidase (NAAA) is also known to inactivate AEA, being found primarily in immune cells[154], including microglia[155], where it thought to be involved in the control of inflammatory processes.

Like AEA, 2-AG is also metabolized into AA by several different enzymes[81,156]. The primary hydrolytic enzyme involved in 2-AG inactivation is monoacylglycerol lipase (MAGL)[156], as shown by the marked increases in 2-AG concentration that are observed in $MAGL^{-/-}$ animals[157]. Unlike FAAH, MAGL is located in presynaptic neurons, close to cannabinoid receptors[151], highlighting its role in controlling eCB mediated retrograde signaling[158]. More recently two other enzymes have been discovered to play lesser, but still

significant, roles in 2-AG inactivation: Alpha/beta-hydrolase domain-containing 6 (ABHD6), located postsynaptically[159], is responsible for the hydrolysis of approximately 5% of 2-AG in the mouse brain[160]. Due to the fact that its active site is located intracellularly[159,160] it is thought that the role of this enzyme is to limit the intracellular concentrations of 2-AG[159]. On the other hand, Alpha/beta-hydrolase domain containing 12 (ABHD12) is a postsynaptic integral membrane protein, whose active site is located on the extracellular domain[159,160]. This enzyme is responsible for the hydrolysis of approximately 9% of 2-AG, and is thought to be important in the control of 2-AG levels in situations where MAGL is not available, or when 2-AG concentrations reach high levels[161].

2.2 – Endocannabinoid System Functioning

The ECS is involved in diverse physiological roles, primarily through its modulatory effect on synaptic function. This modulatory effect, which can be either short- or long-lasting, is achieved through three separate types of eCB signaling: retrograde signaling, non-retrograde signaling and neuron-astrocyte signaling. In addition, there is a persistent, activity-independent, eCB tone[162,163].

2.2.1 – Retrograde Signaling

The first described method of action of the ECS was that of retrograde signaling[164]. Indeed, even after the discovery of other types of ECS signaling, this is still the most researched, with eCBs being, by and large, the most well characterized retrograde messengers. This type of ECS signaling is involved in both short- and long-term synaptic plasticity, by inducing short- and long-term depression, respectively.

2.2.1.1 – Endocannabinoid Mediated Short-Term Depression (eCB-STD)

Endocannabinoid mediated short-term depression (eCB-STD) is a group of phenomena whereby postsynaptically synthetized eCBs are released into the synaptic cleft, to diffuse backwards and activate presynaptic CB_1R, leading to the transient inhibition of either the excitatory or the adjacent inhibitory inputs, through the inhibition of presynaptic VGCC[165]. This group of phenomena can be broadly divided into three groups as a function of the stimuli triggering eCB synthesis: 1) Ca^{2+} increases; 2) receptor activation or; 3) a combination of both:

Firstly, eCB-STD depending on Ca^{2+} driven eCB release, includes depolarization induced suppression of inhibition (DSI) and excitation (DSE), as well as presynaptic suppression caused by N-methyl-D-aspartate receptor (NMDAR) mediated Ca^{2+} influx[165]. In DSI[166] and DSE[167] postsynaptic depolarization leads to Ca^{2+} influx through VGCC, triggering the synthesis of DAG, through a yet unknown enzyme, which will then be converted to 2-AG via DLGα[165]. NMDAR dependent eCB-STD[168] is thought to work in a mechanistically similar way to DSI/E, with the difference being that the increase in postsynaptic Ca^{2+} influx is thought to be mediated by NMDAR, instead of VGCC[165].

Secondly, eCB-STD dependent on receptor driven eCB release[165] – also known as metabotropic-induced suppression of inhibition/excitation (MSI/MSE)[169] – occurs independently of changes in postsynaptic intracellular Ca^{2+} concentration[165,170]. In this type of short-term depression, strong activation of $G_{q/11}$-coupled postsynaptic receptors (e.g., group I mGluRs)[171] leads to the activation of the PLCβ-DLGα pathway, culminating in the synthesis and retrograde release of 2-AG[165,170].

Finally, eCB-STD depending on Ca^{2+} assisted receptor-driven eCB release[165] occurs when subthreshold activation of postsynaptic $G_{q/11}$ coupled receptors (i.e., not strong enough to trigger MSI/MSE) is simultaneously combined with subthreshold increases in intracellular Ca^{2+} concentrations, leading to 2-AG synthesis through the PLCβ-DLGα pathway[84,172,173].

2.2.1.2 – Endocannabinoid Mediated Long-Term Depression (eCB-LTD)

eCB-LTD is a widespread phenomenon, having been described in numerous brain regions and on both inhibitory and excitatory synapses[165,174].

Like eCB-STD, eCB-LTD requires the postsynaptic synthesis and release of eCBs. Indeed, it is thought that the molecular processes for eCB synthesis are similar between short- and long-term forms of eCB mediated plasticity, with eCB-LTD having been found to occur after Ca^{2+} driven, receptor driven, and Ca^{2+} assisted receptor driven eCB synthesis[165,174].

However, eCB-STD and eCB-LTD differ in two critical aspects: while eCB-STD is induced with brief CB_1R activation, induction of eCB-LTD requires several minutes of persistent receptor activation[174,175]. Moreover, CB_1R activation by eCBs, even if prolonged, is not – by itself - enough to induce eCB-LTD, instead requiring simultaneous presynaptic depolarization and increased intracellular Ca^{2+} concentration[176]. The combination of these two events is thought to lead to a shift of the presynaptic

phosphorylation/dephosphorylation equilibrium, towards the latter[170]: on the one hand, CB_1R activation leads to decreased cAMP-PKA activity, resulting in diminished phosphorylation of a yet unknown protein target[170,174], that is thought to result in a reduction in the activity of the active zone protein RIM1α[177] and – at least in inhibitory synapses – the vesicular protein Rab3B[178]. On the other hand, the simultaneous presynaptic firing results in increased presynaptic Ca^{2+} concentrations – through VGCC or NMDAR mediated influx, or release from internal stores[174] – which further reduce the phosphorylation of the aforementioned protein target, by activating the Ca^{2+}-sensitive phosphatase calineurin[176]. Additionally, some authors have proposed that reduced expression of presynaptic P/Q type VGCC, might be an alternative or complementary mechanism for eCB-LTD[179].

2.2.2 – Non-Retrograde Signaling

Although retrograde signaling is the primary mode of eCB action, it is not the only one. Indeed, eCB non-retrograde signaling also exists, with two main forms known – TRPV1-LTD and Slow Self-Inhibition (SSI).

TRPV1-LTD is a type of eCB mediated LTD that is independent of both CB_1R and CB_2R, resulting, instead, of the activation of TRPV1[180–182] by AEA, which acts as a full agonist at this receptor[86]. In this type of LTD, postsynaptic activation of mGluR5 leads to AEA synthesis, through PLC activation, and release[180–182]. AEA then activates postsynaptic TRPV1, leading to Ca^{2+} influx that will – via the activation of calcineurin/dynamin[180] – lead to the endocytosis of postsynaptic AMPAR. It should be noted that, in some brain regions, there is also evidence of a presynaptic component, whereby released AEA also activates presynaptic CB_1R resulting in inhibition[183].

On the other hand, SSI is a type of eCB non-retrograde signaling – found in cortical interneurons[89], pyramidal neurons[90], and in cerebellar basket cells[184] – that relies on activation of postsynaptic CB_1R. In this type of signaling high postsynaptic stimulation leads to increased Ca^{2+} concentrations, that trigger 2-AG synthesis[89]. Once released, 2-AG activates somatic CB_1R which, through $G_{\beta\gamma}$ interactions with GIRK, lead to increased K^+ conductance and neuronal hyperpolarization[89].

2.2.3 - Neuron-Astrocyte Signaling

Neuron-astrocyte eCB signaling has recently become a topic of heavy interest, due to the fact that through this type of signaling, eCBs have been found to be able not only of inducing long-term depression of synaptic activity, but also to be able of inducing short- and long-term potentiation at heteroneuronal synapses (i.e., synapses of adjacent neurons with which there is no direct communication)[185]. Thus, the canonical conception of eCBs as retrograde negative modulators of synaptic activity has, through these findings, come into doubt. Furthermore, it is worth noting that, while traditional eCB signaling is spatially constrained, neuron-astrocyte signaling has been demonstrated to have a much greater reach[185], such that it might be able to influence activity of a broader number of synapses.

2.2.3.1 –Neuron-Astrocyte Mediated Short-Term Potentiation

Neuron-astrocyte mediated short-term potentiation (also known has lateral potentiation)[185] at heteroneuronal synapses has been demonstrated to often co-occur with DSE at homoneuronal synapses (i.e., synapses with which there is direct communication). In short, 2-AG synthetized and released by the homoneuronal postsynaptic neuron will not only activate homoneuronal presynaptic CB_1R (thus inducing DSE), but also astrocytic CB_1R[92]. Unlike neuronal CB_1R, the astrocytic variant of this receptor is coupled to $G_{q/11}$ protein[92,186], such that its activation leads to increased intracellular Ca^{2+} concentrations and subsequent release of glutamate at heteroneuronal synapses[187]. At this synapse, glutamate will simultaneously activate presynaptic mGluR1 – leading to enhanced glutamate release, via increased Ca^{2+} concentrations – and postsynaptic NMDAR – increasing Na^+ and Ca^{2+} conductance, depolarizing the postsynaptic neuron. This combination of events results in a transient potentiation of synaptic activity[187].

2.2.3.2 – Neuron-Astrocyte Mediated Long-term Plasticity

Neuron-astrocyte signaling has also been shown to be involved in some forms of both long-term depression and long-term potentiation[185].

In terms of LTD, this type of signaling has been implicated in a form of spike-timing dependent LTD (t-LTD) whereby back-propagating action potentials trigger the synthesis and release of eCBs, which activate astrocytic CB_1R, leading to astrocytic release of glutamate at the homoneuronal synapse[188]. Activation of presynaptic NMDAR leads to depolarization of the presynaptic neuron[188], and fulfills the

requirements for t-LTD (i.e., that presynaptic firing succeeds, rather than precedes, postsynaptic firing)[189]. Additionally, neuron-astrocyte signaling has also been proposed to play a role in LTD induced by exogenous cannabinoids, whereby CB_1R mediated astrocytic glutamate release activates postsynaptic NMDAR, consequently leading to the internalization of postsynaptic AMPAR[91]. Furthermore, this type of signaling seems to be critically involved in the adverse effects of exogenous cannabinoids on memory – given that, while knockout of neuronal CB_1R had no impact on the severity of these effects, knockout of astrocytic CB_1R effectively abolished them[91].

More recently, neuron-astrocyte signaling has also been found to play a role in a form of eCB mediated LTP at heteroneuronal hippocampal synapses[190]. For this phenomenon to occur, three conditions have to simultaneously be met: eCB activation of astrocytic CB_1R, leading to glutamate release at the heteroneuronal synapse, synthesis and retrograde release of nitric oxide (NO) from the heteroneuronal postsynaptic neuron and, finally, activation of presynaptic mGluR1 and, subsequently, of PKC[190].

2.3 – The Endocannabinoid System in Affective Functioning

Ever since it was first described, and taking into account the fact that cannabis is used recreationally for its mood elevating effects, the ECS has been investigated as to the role it plays in affective functioning. Indeed, not only is the ECS highly expressed in areas classically related to affective and reward processes – such as the hippocampus and the pre-frontal cortex (PFC)[191] – but there are two other complementary lines of evidence supporting the assertion of a role for the ECS in these processes:

On one hand, there is significant evidence that impaired eCB signaling is tied to negative changes in affect[191]. Indeed, animal studies have reported that the selective inactivation of CB_1R (through CB_1R antagonism or deletion) leads to changes at both the behavioral (e.g., anhedonic-like behavior[192], increased anxiety[193], and decreased appetitive behavior[194]) and biological levels (e.g. diminished brain derived neurotrophic factor [BDNF] signaling, and increased hypothalamic-pituitary-adrenal axis [HPA] activity[195–197]) which are typically associated with depressive and anxiety disorders. In addition, studies analyzing animals exposed to manipulations that induce depressive/anxious-like phenotypes (e.g. chronic mild stress [CMS]) have found reduced expression of CB_1R in brain regions known to play roles in affective functioning, such as the hippocampus, hypothalamus and the striatum[198,199], and overall decreased

concentrations of AEA[199–201]. Furthermore, there is human evidence of association between disrupted eCB signaling and affective disorders. The most notable of these pieces of evidence is the fact that the CB_1R antagonist/inverse agonist rimonabant – originally marketed as an anti-obesity drug – had to be removed from the market, after patients with no previous history of psychiatric illness, began reporting anxiety and depressive/suicidal ideation as side effects[31,202]. Accordingly, not only have reduced levels of eCBs been reported in women diagnosed with major depressive disorders[203,204], but there is evidence that certain single nucleotide polymorphisms, which are more prevalent among individuals with mood disorders[205], in the *Cnr1* gene (which encodes CB_1R) are tied to increased risk of stress-precipitated depressive episodes[206] and resistance to antidepressant drug treatment[207].

Secondly, and complementarily to the deleterious effects of ECS impairment, there is evidence that restoring or improving eCB signaling leads to the opposite effect – i.e., improvement of affective state[191]. Indeed, animal studies have found that both exogenous and endogenous cannabinoids, as well as inhibitors of FAAH (which lead to increased AEA levels) reliably lead to both improved outcomes in behavioral assays of depression/anxiety-like behavior[208–212], and to biological changes typically associated with antidepressant effects, such as suppression of stress induced HPA axis activation[196], increased hippocampal neurogenesis[213], and augmented BDNF levels[104]. Moreover, both exogenous and endogenous cannabinoids have been demonstrated to be able of exerting inhibitory effects over monoamine uptake[214] and to be able to directly affect the activity of serotonin (5-HT) and noradrenaline (NA) releasing neurons[209,210,212] – thus providing a possible mechanism for their reported antidepressant effects.

As such, and despite the fact that there is still much to be fully understood about the subject, there seems to be reasonable evidence to assert that the ECS plays a significant role in the control of affective functioning in both animals and human beings.

3 – Adolescence and Chronic Cannabinoid Abuse

3.1 – Adolescence

Adolescence is a critical developmental period, during which mammals transition from childhood into adulthood, occurring between the ages of 12 and 18 years of age in humans, and postnatal days (PND) 28-42 in rats[215]. This period is characterized by extensive biological changes (such as puberty), one of the

most prominent of which is the widespread remodeling that occurs in brain, including increased neuronal plasticity, synaptic sprouting and pruning, extensive myelination (resulting in increased white matter and decreased grey matter volumes), as well as changes in the receptor expression profiles and in neurotransmitter concentrations[216]. These changes are paralleled at the behavioral level, with reported increases in social activity, novelty seeking, and risk-taking behaviors (such as drug use) occurring during this period[215].

Given the heightened plasticity of the brain at this developmental stage, adolescence is also a period of extreme vulnerability, during which external influences (e.g. cannabinoid use) can not only leave lasting impacts, but do so in a much faster and profound manner[217]. Indeed, there is evidence that not only do adolescents become addicted faster than adults[218], but that these addictions are more persistent[219].

With the ECS being known to play important roles in the development of the brain, and adolescence being one such period, it is not at all surprising that the ECS has been found to be involved in the some of the changes that take place during adolescence, such as those observed in HPA activity[220]. Indeed, there is suggestive evidence that ECS functioning may underpin for some of the behavioral changes that characterize adolescence: manipulation of the *Cnr1* gene, meant to induce an increase in receptor-functionality, led to adult rats exhibiting an adolescent-like behavioral profile that was reversed by low-doses of the CB_1R antagonist/inverse agonist rimonabant[221]. In accordance, it has also been demonstrated that wild-type (WT) adolescent mice, pre-treated with the CB_1R antagonist/inverse agonist AM251, do not show the adolescent-typical increased consumption of both alcohol and sucrose, behaving similarly to adults[222].

In addition, the ECS itself has been shown to undergo substantial changes during adolescence: levels of AEA increase from early to late adolescence (although with some region specific fluctuations) in brain areas tied to mood, reward and cognition – such as the PFC, the amygdala and the nucleus accumbens (NAc)[223,224] – with these increases being paralleled by decreased FAAH activity in many of these same regions[224]. Moreover, in the NAc and the PFC there is a decrease in the levels of 2-AG, from early to mid-adolescence, that in the PFC is reversed during late-adolescence[223]. Furthermore, the expression of CB_1R also changes over time, such that receptor density increases gradually from the early post-natal period, peaking at the onset of adolescence, and then gradually decreases towards adulthood[223,225], with some

differences between male and female rats[225]. In addition, the changes at the molecular level might have some parallel in functional terms in the hippocampus, such that both eCB-LTD and the inhibitory effect of THC over synaptic transmission, are increased in adolescent rats in comparison to adult rats[226].

3.2 – Effects of Chronic Adolescent Cannabinoid Abuse

Given that there seems to be a strong relationship between eCB signaling and the modifications occurring in the CNS during adolescence, it is reasonable to expect that cannabinoid use within this period, during which it typically begins[215], might interfere with the changes taking place, leading to lasting alterations. Accordingly, there is significant evidence to support this idea, with respect to both cognitive and affective functioning, obtained from both human and rodent studies.

3.2.1 – Human Studies of Chronic Adolescent Cannabinoid Abuse

Most human data regarding the lasting effects of chronic adolescent exposure to cannabinoids have been obtained by observational studies. While these studies do find some significant associations between adolescent cannabinoid use and negative outcomes – in both the cognitive and affective domains – the interpretability of the results is necessarily hindered by the problem of inverse causality: that is, there is a possibility that cannabinoid abuse might be the result, and not the cause, of the changes found. Moreover, cannabinoid abuse and these negative outcomes may not be causally related, but – instead – be symptoms of higher level causes, such as socioeconomic disadvantages or poor family relationships[227].

The fact that, in epidemiological studies, adolescent cannabis use has been consistently associated with poorer school performance and earlier school abandonment[228–231] has led some researchers to propose a causal relationship between the two. One often cited mechanism for this is the so-called amotivational syndrome, which is purported to be characterized by "apathy and diminished ability to concentrate, follow routines, or successfully master new material"[232], and whose neurobiological basis has been proposed as being related to cannabis-induced impairments in motivation and reward circuitry[233]. The existence of this syndrome is, however, subject to wide discussion, with no conclusive evidence having emerged thus far[232]. Another such suggested causal mechanism relates to the proposed intelligence quotient (IQ) lowering effects of cannabis use, whereby lack of educational achievement would be propitiated by reductions in intelligence[234]. This proposal, however, seems to not be supported by data, as other studies have found no

effect of cannabis use on IQ[235], especially when adequate adjustments are made for socioeconomic factors[236]. Indeed, a more likely explanation for this apparent deleterious effect of cannabis on educational performance, is the existence of the aforementioned common psychosocial factors, namely socioeconomic disadvantage and lack of parental educational achievement[227,229,231].

Despite this, there is significant evidence suggesting that individuals, who regularly used cannabis as adolescents, present alterations at the structural, functional and neurocognitive levels. Structurally, it seems that adolescent cannabis use leads to decreases and increases in gray and white matter[237,238], respectively, as well as to changes in cortical thickness, in a number of brain regions (including the PFC)[239], and to decreases in both hippocampal[240] and orbitofrontal cortex (OFC) volumes[241]. Moreover, it has been reported that adolescent cannabis use leads to decreased white matter integrity, with age of regular drug use onset being a key predictor of white-matter tract microstructural anomaly severity[242].

In accordance with these structural abnormalities, research looking at functional activity, has found it to be altered in the brains of individuals who used cannabis as adolescents[238]. Specifically, abnormal activation patterns have been found in the limbic and parietal regions, as well as in the cerebellum and PFC of these individuals[243–247]. Likewise, reductions in blood flow have been observed not only in prefrontal brain regions, but in temporal and insular regions as well[248]. Furthermore, one interesting study found that, despite showing normal performance in a cognitive task, participants who had used cannabis as adolescents showed abnormally high prefrontal activation, suggesting a need for compensatory recruitment of neuronal resources to achieve normal performance[247]. In addition, resting-state activity patterns have also been shown to be significantly different between adolescent cannabis users and control participants[238].

In line with the structural and functional abnormalities described above, cognitive studies have found adolescent users to present impaired performances in tasks relating to working memory and executive functioning[243,244,249,250], attentional control[251,252], cognitive inhibition and impulsivity[249,252], decision-making[245,252], and to both visuospatial[253] and verbal memory[240]. Furthermore, the severity of these impairments is related to both the age at use onset, as well as to the frequency of use and to the doses used[243,249].

While the lasting cognitive effects of adolescent cannabis use have been relatively extensively investigated through several approaches, almost all studies on the affective consequences[b] of such drug exposure have been epidemiological in nature, although many of the functional and structural alterations reported above[238] also impact brain regions heavily involved in affective functioning.

With regard to anxiety, a number of studies have found adolescent cannabis use to be a risk factor for the later development of anxiety disorders, with some studies reporting these individuals to be more than twice as likely to be diagnosed with an anxiety disorder as adults, and to be at even greater risk if cannabis use began before age 15, and if consumers are female[254–256]. It should, however, be noted, that a recent meta-analysis[257] of several epidemiological studies concluded that, depending on whether all studies or only high-quality ones were included in the analysis, adolescent cannabis use either represented a negligible risk factor for, or had no influence on, adult likelihood of anxiety disorder diagnosis, respectively.

Concerning depressive disorders, on the other hand, studies have found that adults, who were cannabis users as adolescents, are at an increased risk of being diagnosed with a depressive disorder, with risk being higher for females than males[254–256,258]. Underlining this apparent deleterious effect of adolescent cannabis use on adult affective functioning, it has been reported that this type of drug use leads to a significant increase in the risk of suicidal ideation and/or suicide attempts, which – again – is greater for females, and in proportion to both frequency of use, and age of use onset[258]. Furthermore, the results of a twin study[259] suggest that at least some of these effects are attributable to genetic vulnerability, and not merely to environmental causes.

Thus, while there seems to be significant evidence to support the assertion that adolescent cannabis use induces lasting deleterious effects on affective functioning – especially in the female population – these data are inherently limited by their observational nature. As such, one must complement human data with animal pre-clinical research, so as to better understand the relationship between adolescent cannabinoid abuse and affective disorders.

[b] Because the present work focuses on the effects of chronic adolescent cannabinoid exposure on anxiety- and depressive-like behaviors, studies on the possible relationship between cannabis use and the development of psychotic disorders will not be approached.

3.2.2 – Animal Studies of Chronic Adolescent Cannabinoid Abuse

Unlike human data, animal studies are not susceptible to reverse causality, and are less vulnerable to the possible existence of uncontrolled (or uncontrollable) variables that might influence the results – such as the potency of cannabis used, the accuracy of subject reports, or other lifestyle choices such as concomitant use of other psychoactive substances. Indeed, there is ample animal behavioral data supporting the view that the chronic adolescent use of cannabinoids leads to persistent changes in both cognitive and affective functioning[260]. Moreover, these behavioral studies are also accompanied by significant evidence of biochemical and electrophysiological changes, as a consequence of adolescent treatment, thus giving some evidence of the biological substrates underlying the observed behavioral alterations.

Furthermore, it is worth nothing that, in studies that compare the same drug administration schedule in adolescent and adult rats, it is often the case that the changes induced by drug exposure are only present in the former group, with the latter showing either no or opposite effects[261,262] – again underlining the special vulnerability of the adolescent brain to the effects of cannabinoid abuse. Alternatively, this may also suggest that, at more advanced ages, there is a decline in ECS functioning, that treatment may be offsetting[263].

3.2.2.1 – Cognitive Effects

The cognitive domain, where the effects of chronic cannabinoid exposure during adolescence have been most amply studied, is that of memory. The majority of studies (although exceptions do exist[264–268]) have demonstrated that prolonged administration of cannabinoid receptor agonists (CBRAs), such as THC, WIN55,212-2, CP 55,940 or HU-210 (fig. 1.3; table 1.1), induces persistent impairments in recognition memory, as indexed by the novel object recognition test (NORT)[261,269–282]. These impairments are often accompanied by spatial memory deficits in the novel object place recognition test (NOPRT)[268,269,272,277,281–283]. However this deleterious effect seems to be task-specific, as the majority of studies using both the Morris water maze (MWM)[265,283–286] – the gold-standard for testing hippocampal-dependent spatial memory[287] – and the active place avoidance paradigm (APAP)[284,288] have found no significant changes in this domain of memory, as a consequence of chronic adolescent CBRA exposure.

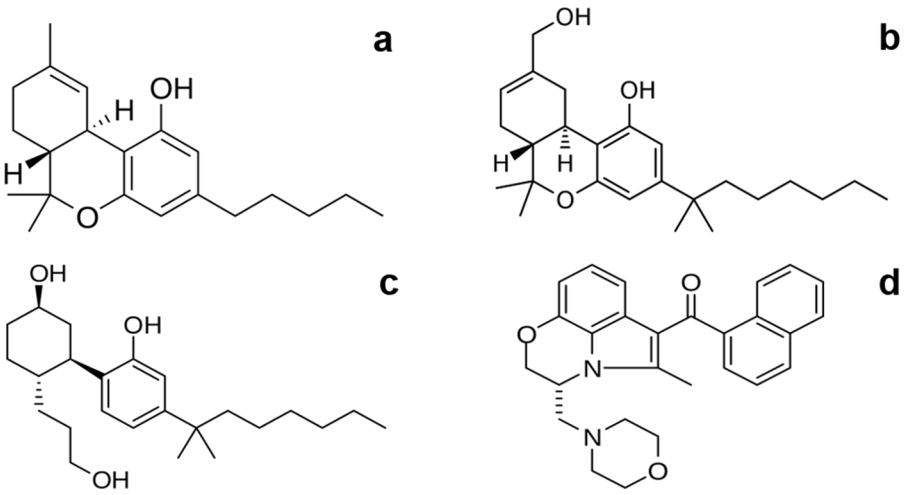

Fig. 1.3 – Molecular structures of the four CBRAs most used in research. All research on the long term effects of chronic adolescent cannabinoid exposure has been performed with one of four non-selective CBRAs: THC **(a)** is a partial agonist with equal affinity for CB_1R and CB_2R, HU-210 **(b)** is a potent full agonist at both CBRs, with one order magnitude greater selectivity for CB_1R, CP 55,940 **(c)** is a full agonist at both CBRs with equal relative affinity for both, and WIN 55,212-2 **(d)** is a full agonist at both CBRs with slightly greater selectivity for CB_2R.

Regarding working memory, studies using either the Y-maze, the T-maze or the radial maze, reported that chronic adolescent CBRA exposure leads to persistent deficits in spatial working memory[272,289–291]. Complementing reports of these deficits, Gomes et al.[292] showed that the attentional control of rats chronically treated with CBRAs during the adolescent period showed signs of impairment in relation to vehicle treated controls. As such, these combined results suggest that chronic adolescent CBRA exposure leads to altered adult executive functioning.

It is of note that, in contrast to what is reported at the levels of affective functioning, the deleterious effects of adolescent CBRA exposure on cognitive function are remarkably similar between sexes, with no clear trend emerging for sexual dimorphism.

Table 1.1 – Pharmacological characteristics of the four CBRAs most used in research.

Drug	Efficacy	CB_1R		CB_2R	
		K_i (nM)[293]	EC_{50} (nM)[10]	K_i (nM)[293]	EC_{50} (nM)[10]
Δ^9-THC	CB_1R/CB_2R partial agonist	5.05 – 80.3	9.1 – 530	3.13 – 75.3	41.8 – 1000
HU-210	CB_1R/CB_2R full agonist	0.06 – 0.73	0.02 – 7.2	0.17 – 0.52	0.37 – 1
CP 55,940	CB_1R/CB_2R full agonist	0.5 – 5	0.8 – 310	0.69 – 2.8	0.72 – 2.89
WIN 55,212-2	CB_1R/CB_2R full agonist	1.89 – 123	7.6 – 410	0.28 – 16.2	0.41 – 3

K_i, inhibitory/dissociation constant (measure of drug affinity for a given receptor); EC_{50}, half maximal effective concentration (measure of drug potency for a given receptor).

3.2.2.2 – Effects on Anxiety-like Behavior

Contrary to the observed cognitive effects of adolescent CBRA exposure, the results regarding the consequences of this type of treatment on adult anxiety-like behavior show more heterogeneity, with some contradictory and/or test-specific findings being reported.

When animals chronically exposed to CBRAs during adolescence are tested in the elevated plus maze (EPM) at adulthood, the majority of studies has found no significant treatment effects[210,264,265,272,290,294–298]. However, there are reports of CBRA-treated rats showing either increased[299] or decreased levels of anxiety-like behavior[300–302] in this test. While the reason underlying these differences is not clear, it is likely that differences regarding animal strains, and dosing/testing schedules are the primary causes.

Similarly, studies using the open field test (OFT) generally report that animals chronically exposed to CBRA treatment during adolescence show no significant behavioral differences from controls in this test – i.e., no changes in either anxiety-like and/or locomotor-related behavior[210,269,283,294,300,303]. Nonetheless, there is a limited number of reports describing changes pointing to both increased[271,304] or decreased[296,301,305] anxiety, as well as changes in locomotor behavior[278,306]. Moreover, unlike in the case of the EPM, some of these discrepant results are found in studies employing the exact same strains and dosing/testing schedules effects[278,294], thus further difficulting the determination of a clear trend in effects.

While the EPM and the OFT are the most widely used behavioral paradigms to measure anxiety-like behavior, they have some significant shortcomings (see chapter 2). However, a small number of studies has employed other assays of anxiety-like behavior: while studies with the holeboard test (HBT) find no effect of chronic adolescent CBRA exposure on adult anxiety-like behavior[271,307], studies using the light-dark box (LDBT)[278,306] and the novelty suppressed feeding tests (NSFT)[210], find that CBRA-treated rats show increases in anxiety in comparison to vehicle-treated controls.

Thus, based on these findings, a relatively unclear picture emerges. While the majority of studies do not find any effect of adolescent CBRA use on adult anxiety-like behavior, there is a non-insignificant amount of studies pointing to the possibility of other effects. As such more data should be obtained to fully elucidate the effects of chronic adolescent CBRA exposure.

3.2.2.3 – Effects on Mood and Reward Signaling

While the effects of adolescent CBRA treatment on anxiety-like behavior are somewhat contradictory, the same cannot be said for the effects observed on the social behavior of rats. Indeed, it has been consistently reported that rats chronically exposed to CBRAs during the adolescent period show persistent deficits in social interaction and social motivation at adulthood. In the majority of studies using the social interaction test (SIT) CBRA-treated rats choose to spend significantly less time interacting with an unknown same-sex partner, than control rats[261,262,262,273,274,276,282,308]. Likewise, authors using the social motivation and social novelty task (SMSNT) have reported decreases in both the interaction with, and preference for, novel social partners[278,290,306], as a lasting consequence of adolescent CBRA treatment.

However, these reports can be interpreted in several ways, given that measures of social behavior straddle the line between measuring anxiety and reward signaling and/or mood function[309]: one can interpret reduced social interaction and social motivation as being due to either increased anxiety, decreased reward value for social stimuli (known to be highly rewarding to rodents[310]) or even due to a combination of both. Indeed, a compelling case can be made for interpreting these deficits as resulting from decreases the hedonic value of social behavior, given that they are a) independent of changes anxiety-like behavior (as SIT results do not correlate to those of other anxiety tests[311]) and b) often associated with changes in other tests, where impaired reward signaling and/or mood functioning[276] are found.

The changes in reward signaling have been demonstrated in several ways: Schneider and Koch[279] were the first to report that adult rats chronically exposed to WIN55,212-2 during the adolescent period, had lower "break points" on a progressive ratio operant task, indicating lower motivation to obtain a reward. In line with this, Chadwick et al.[312] reported that female rats chronically exposed to CP 55,940 during adolescence show persistent decreases in motivation to engage in sexual behavior (which is accompanied by other changes in sexual behavior[313]). However, the most compelling evidence of impaired reward signaling, as a result of protracted adolescent CBRA exposure, comes from studies employing the sucrose preference (SPT) and palatable food preference tests (PFPT): in most of these studies (but see references[269,298,312]) the CBRA-treated groups show decreased intake of, or preference for, either a sucrose solution[210,276,294,296] or a palatable food[276] when compared to control rats, suggesting that these CBRA-

treated rats are less sensible to the rewarding properties of these stimuli[312] – a result that is generally interpreted as being indicative of anhedonia, a key feature of depressive disorders[314].

Further underlining the similarities between the changes induced by chronic adolescent CBRA exposure and the features of depressive disorders, rats subjected to manipulations of this sort have been reported to show behavioral profiles in the original and modified versions of the forced swim test (FST and mFST, respectively) that are associated with depressive-like behavior. Specifically, in most reports (but see references[268,269]) CBRA-treated rats show increased immobility times[210,276,280,282,294,308,315], decreased swimming times[210,276,280,282,308,315] and reduced latency to first immobility[210] in comparison to vehicle-treated controls, suggesting a lasting impairment in stress-coping behavior.

In sum, there is enough animal behavioral data to support the assertion – suggested by human observational data – that chronic adolescent CBRA exposure induces persistent deleterious changes in reward signaling and mood that are similar to those found in depressive disorders.

3.2.2.4 – Biochemical and Morphological Effects

Given the extensive and lasting impact that chronic adolescent treatment with CBRAs has on both cognitive and affective functions, it is reasonable to expect these changes to have biochemical correlates. Indeed, numerous studies have confirmed this to be the case, in a myriad of systems and brain regions.

In the glutamatergic system, changes have been reported to occur primarily in the hippocampus and the PFC: At the level of the hippocampus, Rubino et al.[316] found that adult male rats treated with escalating doses of THC during adolescence, showed diminished NMDAR density at adulthood. However, two posterior studies[280,317], found this same effect to be observable only in female rats. In addition, other reports found reduced levels of mGluR5 [318] and increased levels of NMDAR subunit 2B (GluN2B) and AMPAR subunits 1 and 2 (GluA1 and GluA2)[268], as well as a decrease in the levels of K^+-evoked glutamate release[318]. In the PFC, adolescent treatment with CBRAs was found to lead to both decreased levels of mGluRs 2 and 3, and reductions in the co-localization of CB_1R with vesicular glutamate transporter 1 (vGluT1) in the medial portions of this region (mPFC)[305], as well to as time dependent increases in the levels of both NMDAR subunit 2A (GluN2A), GluN2B and GluA1 subunits[291]. Moreover, in a study focusing on the effects of chronic adolescent CBRA exposure on prefrontal gene expression, numerous

time-dependent decreases were found in the expression of genes relating to several glutamate receptor subunits[308].

Regarding the GABAergic system, a picture emerges that is somewhat the mirror image of the one found for the glutamatergic system. Indeed, it has been proposed that chronic adolescent exposure to CBRAs disrupts the excitation/inhibition equilibrium in the hippocampus and PFC in opposite manners, by tilting this equilibrium towards inhibition in the former region, and towards excitation in the latter[317]. Indeed, in the hippocampus of chronically treated animals, authors have found treatment to lead to persistent increases not only in K^+-evoked GABA levels, but also in the density of GABA A and B receptors ($GABA_AR$ and $GABA_BR$, respectively), as well as in decreased levels GABA transporter 1 (GAT-1) RNA [317]. Contrastingly, studies focusing on the PFC have found that CBRA treatment leads to lasting decreases in the expression of the GABAergic system in this region: Zamberletti et al.[281] reported decreases in both prefrontal levels and activity of glutamate decarboxylase 67 (GAD67) – the main synthetic enzyme for GABA – as well as decreased levels of GABA, as a result of treatment with escalating doses of THC. In addition, a pair of key studies[278,306] replicated these deficits in GAD67, and found the changes in prefrontal GABAergic signaling to be associated with changes in the frequencies and firing patterns of both mPFC pyramidal neurons and ventral tegmental area (VTA) DA neurons. Moreover, by showing that intra-mPFC administration of a selective $GABA_AR$ agonist (muscimol) restores performance in tasks relating to object recognition memory, social motivation and anxiety to control levels[278], these authors conclusively demonstrated that disruption of frontal GABAergic signaling is responsible – either directly or through influence on DA signaling – for at least some of the behavioral deficits, induced by chronic adolescent CBRA exposure. Finally, similarly to what was observed in the glutamatergic system, analysis of prefrontal GABAergic system-related genes, detected time-dependent decreases in the expression of several genes coding for both subunits of both GABA receptors, as well as for enzymes involved in the synthesis of GABA[308].

The changes reported by Renard et al.[278,306], at the level of VTA DA neuron firing, are consistent with previous evidence, showing that not only does chronic exposure to CBRAs lead to changes in the activity of DA neurons[292], but also that it induces other changes in the DAergic system. Indeed, previous studies found CBRA treatment to lead to increased NAc density of DA receptor type 1 (D_1R)[280,319] and D_2R[280], as

well as to a – male specific – increase in prefrontal D_2R density[280]. In the CA1 region of the hippocampus, however, treatment led to a decrease in D_2R density[319]. Moreover, treatment also led to female specific increases in DA transporter (DAT) levels in the caudate-putamen (CPu)[319] and to augmented DA metabolism in striatal regions[264] (in a study using only male rats), as well as to diminished density VTA tyrosine hydroxylase (TH) expressing cells[320]. In agreement with these changes, other authors have reported that animals that underwent chronic adolescent exposure to CBRAs show heightened behavioral responses to amphetamine[292,321] – suggesting that this CBRA treatment may lead to sensitization of the DA system.

In comparison to the DA system, research on the effects of persistent adolescent exposure to CBRAs on other monoaminergic systems has been relatively sparse. This is the case of the NA and 5-HT systems, even though both are highly relevant in numerous cognitive and emotional processes. Indeed, to date, only a few studies have focused on these systems: Lopez-Rodriguez et al.[322] reported a male specific increase in serotonin transporter (SERT) expression in parietal cortex fibers, as a consequence of chronic adolescent CBRA treatment. In addition, whereas 5-HT type 1A receptor ($5\text{-}HT_{1A}R$) density and mRNA expression were not found to be significant altered after treatment[323], a significant transient decrease was found in the prefrontal expression of the gene encoding for the 5-HT type 2A receptor[308] ($5\text{-}HT_{2A}R$). Thus, and given the paucity of evidence, little can be asserted about the effects that CBRA treatment has on these systems, and how those effects might relate to the behavioral phenotypes observed.

The opioid system has also been heavily implicated on mood functioning and reward signaling[324], and studies have demonstrated that chronic adolescent CBRA exposure induces several alterations in this system. Specifically, while Ellgren et al.[223] found increases in μ-opioid receptor (MOR) function in the substantia nigra (SN) and VTA of treated rats, without concomitant changes in MOR density, Biscaia et al[325] found gender-dependent changes in MOR density in the cingulate cortex, hippocampus, thalamus and subcallosal streak of treated rats (with male and female rats showing decreased and increased density, respectively). These disparities are likely attributable to differences in drug and treatment schedules used. Moreover, using a different drug schedule, Rubino et al.[294] found that treatment of adolescent rats led to female-exclusive increases in the NAc levels of dynorphin A – a κ-opioid (KOR) agonist peptide, known to be involved in the regulation of negative emotions. Finally, two studies have focused on the expression

of proenkephalin mRNA, finding contrasting results (again, likely due to highly dissimilar drug treatment schedules): whereas Morel et al.[326] found decreased expression in both the NAc and the CPu, Tomasiewicz et al.[327] found the opposite effect for the NAc, without observable changes in CPu expression. As such, while there is some compelling evidence of changes to the opioid system, as a consequence of chronic adolescent CBRA exposure, the lack of consistency between reports makes it difficult to conclusively relate these changes to any of the behavioral changes previously described.

Given that CBRAs overwhelmingly exert their effects through interactions with the ECS, it is reasonable to expect that chronic adolescent CBRA exposure would induce lasting changes in this system. Indeed, this seems to be the case at both the receptor, eCB, and degradative enzyme levels.

Regarding the effects of treatment on cannabinoid receptors, studies have obtained somewhat contradictory results, likely as a result of the differences in the technique used: firstly, when receptor protein levels have been analyzed through western blot, results have been inconsistent, with some studies finding that treatment leads to increases in CB_1R protein levels the striatum[328] and the hippocampus[329], while others report no changes in either of these areas[330]. Moreover, while no effect was found in the amygdala of treated mice[297], decreases and increases in CB_1R and CB_2R protein levels, respectively, were found in prefrontal tissue of female rats chronically exposed to THC[282]. On the other hand, when studies have used immunohistochemistry, the majority of, but not all[331], reports have found treatment to lead to no significant alteration in CB_1R expression[305,312,320,322]. Contrastingly, with a few exceptions[223,272,317,326,332], studies using radioligand binding assays – a more precise and quantitative measure of receptor levels – have found that chronic adolescent CBRA treatment leads to significant widespread decreases in CB_1R densities[280,291,294,315,333,334]. Likewise, analyses of CB_1R function have shown that adolescent exposure to CBRAs results in long-lasting reductions in receptor function – suggesting a decrease in receptor sensitivity to ligands[280,291,294,329,332], with only a limited number of studies finding different results[223,317,326].

Regarding eCBs, the results reported have been suggestive of both eCB- and region-specific effects. While Rubino et al.[291] found decreases in PFC AEA levels, Schoch et al.[298] reported increased levels of this molecule in the NAc (after a 6-hour food deprivation period), and Tomas-Roig et al.[303] reported similar increases in the hippocampus of CBRA-treated rats. Conversely, the two reports[298,303] that have measured 2-AG levels, found them to not be altered by CBRA treatment.

Relatedly, the effects of chronic adolescent CBRA exposure over eCB degradative enzymes, have not yet been fully elucidated, with inconsistency in the results of the few studies published so far (likely as a result of differences in the rodent species used, in the gender of the animals, and in both the time of measurement and the technique used for it). Specifically, while Rubino et al.[291] found a persistent decrease in FAAH levels, and a transient decrease in the MAGL PFC levels of treated rats – which is accompanied by a decrease in the expression of the gene coding for the latter enzyme[308] – Lovelace et al.[305] found no alteration in MAGL levels, in this same region, in mice. Additionally, Gleason et al.[318] found the levels of both MAGL and FAAH to be increased in the hippocampus of adult male mice chronically exposed to WIN 55,212-2 during adolescence.

Thus, as would be expected, there is ample evidence supporting the notion that chronic adolescent CBRA exposure induces lasting alterations in the functioning of the ECS, that may underpin the alterations observed when these animals are put through behavioral testing, although more research is clearly needed.

Related to both cognitive as well as affective functioning, neurogenesis has been consistently shown to be impaired by chronic adolescent exposure to CBRAs. Thus, male animals treated with WIN 55,212-2 were demonstrated to have decreased hippocampal neurogenesis[284], while another report showed the hippocampal antineurogenic effects of HU-210 to be male-specific[321]. Moreover, in a recent study using only female rats, chronic treatment with THC led to long lasting decreases in the number of dentate gyrus (DG) immature neurons, suggesting a deleterious effect of treatment[315].

As is the case with neurogenesis, there is consistent evidence that chronic adolescent CBRA exposure induces significant alterations in the morphology of neuronal cells. As such, dendritic length has been found to be reduced in both immature[315] and mature[316] hippocampal neurons, as well as in pyramidal neurons located in layers II/III of the PFC[335]. Similarly, spine density has been shown to be reduced in neurons located in the NAc[336], the hippocampus[316], the amygdala[297], and in PFC layers II/III[291].

Given the alterations in both neurogenesis and neuronal morphology, the cognitive deficits observed as a consequence of chronic adolescent CBRA exposure, and the known widespread involvement of the ECS in plasticity phenomena, it stands to reason that this type of treatment would have correlates at the level of neuroplasticity. Indeed, reports have shown chronic treatment with CP 55,940 induces female-specific decreases in hippocampal BDNF levels[331], while 24-hours after chronic exposure to THC a decrease was

observed in the expression of the gene coding for this neurotrophic factor[308]. Moreover, alterations have been reported in the levels of several proteins known to be key players in the regulation of synaptic plasticity, although some significant contradictions, and gender-dependent differences exist: specifically, reports have found decreases in post-synaptic density protein 95 (PSD95) levels in the PFC of male rats[335] while, in females, authors have reported this protein to be both transiently increased for a period after treatment – being normalized at adulthood[291] – as well as decreased, at a posterior adult time-point[337]. Similarly, in the hippocampus of male rats, two reports from the same group have demonstrated both increases[268] and decreases[316] in the levels of this protein (despite using the exact same protocol), while a third report from another group found no treatment effect[335]. Synaptophysin, another protein known to be involved in synaptic function and learning processes[338], has been shown to be altered in a gender- and region-specific manner, whereby it is increased in the hippocampus of male animals[268] (but see reference[335]), whereas it is decreased in the PFC of females[337]. In the same manner, vesicle-associated membrane protein 2 (VAMP2), a protein involved in the synaptic vesicle docking and fusion[339], has also been shown to be decreased in the hippocampus of male animals[316]. Furthermore, expression of the gene coding for activity-regulated cytoskeleton-associated (Arc) protein – thought to play a critical role in learning and memory processes[340] – has been shown to be transiently decreased 24-hours after chronic adolescent CBRA exposure[308], and was found to be decreased in a female-specific manner in both the hippocampus and PFC of adult animals[271]. Likewise, phosphorylated cAMP response element-binding protein (pCREB), another key player in synaptic plasticity phenomena[341], has been shown to be reduced in the PFC and hippocampus, and increased in the NAc of female, but not male, adult animals that underwent chronic adolescent exposure to THC[294]. Finally, Renard et al.[306] have recently reported that chronic adolescent treatment with CBRAs leads to a marked dysregulation and suppression of the mammalian/mechanistic target of rapamycin complex 1 (MTORC1) pathway – known to be involved in synaptic plasticity and cognitive processing, as well to be altered in numerous neuropsychiatric disorders[342].

In addition to alterations at the level of synaptic plasticity, there is some evidence to support the notion that chronic adolescent CBRA exposure alters brain metabolic activity, although it is not clear in which way. Indeed, despite the fact that Higuera-Matas et al.[325] found female-specific increases in metabolic

activity in the PFC and septal nuclei, and decreased activity in the DG of CP 55,940-treated rats, there is molecular data suggesting that both whole brain pyruvate decarboxylase and NADH dehydrogenase expression[343], as well as PFC levels of several mitochondrial and glycolytic enzymes[337], are reduced by treatment.

In more recent years there has been some interest in the role that neuroinflammation plays in both depressive disorders and in cognitive deficits[344]. Strikingly, it seems to be the case that chronic adolescent exposure to THC, may exert some of its effects through the modulation of inflammatory responses, whereby, in female animals, this type of treatment led to increases in the prefrontal levels of the pro-inflammatory agents tumor necrosis factor α (TNFα), inducible nitric oxide enzyme (iNOS) and COX-2, concomitantly with increases in both the expression of ionized calcium-binding adapter molecule 1 (Iba1; a marker for activated microglial cells), and the number of amoeboid microglia[282] – all of which suggest a lasting pro-inflammatory effect of THC treatment. Curiously, in another study – where male animals underwent similar treatment – both increases in TNFα and iNOS, as well as decreases in the levels of the anti-inflammatory cytokine interleukin-10 (IL-10) were observed in hippocampal (instead of prefrontal) tissue, with no changes being observed in either COX-2 or Iba1[268].

Finally, numerous studies have looked into the effects of chronic adolescent CBRA exposure on other assorted biochemical targets, such as changes in histone methylation and acetylation levels[345], in c-FOS reactivity to several different stimuli[301,346], in the levels of hippocampal polysialylated-neural cell adhesion molecule (PSA-NCAM)[265], and of proteins such as 90 kDa heat-shock protein (HSP90) and its chaperone, the activator of 90 kDa heat shock protein ATPase homolog 1 (AHA1) enzyme[328,330,343], finding this type of exposure to lead to significant alterations.

3.2.2.5 – Electrophysiological Effects

Given the involvement of the ECS in neuroplasticity phenomena, and both the behavioral and molecular evidence pointing to disrupted learning and memory mechanisms, resulting from chronic adolescent CBRA exposure, it is unsurprising that there is consistent evidence of alterations in the electrophysiological domain.

At the single neuron level, alterations have been found in both NA, 5-HT, DA, and mPFC pyramidal neuron activity: Bambico et al.[210] reported CBRA treatment to lead to increased spontaneous and evoked

firing of locus coeruleus (LC) NA neurons, and to the opposite effect on dorsal raphe nuclei (dRN) 5-HT neuron firing. Concerning the DAergic system, two report have found chronic exposure to CBRAs to lead to either no alteration[296] or an increase[292] in spontaneously active DA neurons (likely due to differences in the drug and administration schedule used). Moreover, whereas WIN 55,212-2-[296,347], cocaine-[347], amphetamine- [347] and morphine-evoked[347] DA neuron firing rates were demonstrated to be depressed as a result of adolescent CBRA treatment, an increase was found both in the number of burst firing DA neurons[278], as well as in their firing frequencies and number of bursting episodes[278,306] – suggesting that CBRA treatment induces long-lasting hyperactivity in the DA system[306]. Similarly, when looking at the mPFC, Renard et al.[278] found that the pyramidal neurons, of rats chronically exposed to THC as adolescents, showed increases in their firing frequencies, as well as in the number of spontaneous burst firing episodes, with an increase in the number of burst firing neurons also being observed.

In recent years, there has been an increase of interest in the layer II/III mPFC synapse, as a potential target for the long term effects of chronic adolescent CBRA exposure. In this regard, while Lovelace et al.[305] found no changes in either short-term presynaptic plasticity or basal synaptic efficiency, they did observe a significant impairment of both mGluR2/3 mediated LTD, and eCB-LTD at this site. This latter finding has been corroborated by both Rubino et al.[291] and Cuccurazzu et al.[315]. Furthermore, the latter authors additionally found DG LTP to be impaired, in both mature and newborn neurons[315]. Moreover, not only was LTP in the hippocampus-PFC pathway (a pathway critical for adequate cognitive functioning) found to be impaired as a result of chronic CP 55,940 treatment[335], but, in another study[283], LTP in the ventral subiculum (vSub)-NAc pathway was also found to be transiently diminished, whereas no such effect was found in perforant path (PP)-DG pathway LTP.

Lastly, two studies by Raver et al.[275,348] have looked at the effects of chronic adolescent CBRA exposure on local field potentials (LFP). Specifically, these authors have reported that chronic adolescent exposure induces dose-dependent decreases in the power of gamma (γ), beta (β), alpha (α), and theta (θ) frequencies recorded in the mPFC and the somatosensory cortex, as well as significant decreases in LFPs evoked by the combination of kainic acid and carbachol. Thus, and given that Renard et al.[278], also found mPFC γ frequencies to be altered as consequence of treatment, these data suggest that chronic adolescent CBRA

exposure induces alterations in the functioning of synchronized neural networks, that may underlie some of the behavioral alterations observed in these animals.

3.3 – Sexual Dimorphism in the Effects of Chronic Adolescent Cannabinoid Abuse

One peculiar finding in both the human and animal research literature on the effects of chronic adolescent CBRA abuse, pertains to the fact that there seems to be a significant effect of gender on the severity of drug effects. Indeed, there is evidence that, despite the fact that men are the primary consumers of CBRAs[349,350], and stand at an increased risk of developing CUD[349–351], women show a faster progression from first use to problematic use[350,352], and experience more severe withdrawal effects[350,353–355]. Moreover, it seems to be the case that women are more vulnerable to both the immediate subjective effects of CBRAs[350] as well as to the psychiatric consequences of long-term use, especially when it concerns depressive and anxiety disorders[254–256].

Because human studies, even experimental ones, suffer from several limitations, animal data provides a unique window into the possible gender-dependent effects of CBRAs. Indeed, there is some relevant evidence that female animals are more sensitive to the effects of these drugs – showing greater drug-induced antinociception[350], anxiogenesis[349,350], and locomotor impairment[328,349,350,356,357] – as well as responsive to their reinforcing effects[349,350,358]. Furthermore, there is some suggestion of a greater negative effect of chronic adolescent CBRA exposure in females, with regards to affective behavior, and a roughly equal effect on cognitive function[268,276,282,294,308,315,359].

There is increasing evidence that this dimorphism may arise from a confluence of several factors[349,350,359,360]. There is some evidence suggesting that the ECS of females may differ from that of males[360], with differences having been found in the levels of eCBs[361], in the density[271,280,362], affinity[362], and function[271,280,360] of CB_1R (importantly, in some brain regions critically involved in affective functioning[360]). Furthermore, differences have been found in both the recovery of hippocampal CB_1R expression[334] following protracted CBRA administration, as well as in the CB_1R desensitization response[332] to this type of manipulation. In addition, in a recent study, it was demonstrated that pharmacological blockade of FAAH led to a marked female-specific suppression of inhibitory synaptic transmission, suggesting the existence of gender-specific tonic eCB signaling[363].

Relatedly, the female ECS shows significant variation across the hormonal cycle, via the action of sex steroids such as estrogens and progesterone[349], which correlate to fluctuations in eCB levels[364], as well as in CB_1R density, affinity and function[362,365], and impact behavioral tests[329]. Even more interestingly, and in line with this, there is evidence supporting the notion that ovarian hormones may alter both cannabinoid seeking and taking behaviors[366,367]. Furthermore, while ovarian hormones seem to potentiate the effects of CBRAs, androgens such as testosterone seem to have the opposite effect, reducing the behavioral responses to these drugs[368,369].

Finally, there is convincing evidence supporting the idea that there may be gender-specific pharmacokinetic differences in cannabinoid metabolism that may, at least partially, underlie the stronger effects observed in females[349]. Specifically, while Tseng et al.[370] reported similar levels of THC and its metabolites in males and females, these authors found the levels of these compounds to be higher, and present for longer periods of time, in the brains of females. On the other hand, Wiley et al.[371] found females to have higher brain and blood levels of the CB_1R-active THC metabolite 11-OH-THC. Interestingly, while there is some suggestion that this difference is at least partially independent of hormonal influence, an important role for gonadal hormones also seems to exist, such that testosterone led to a decrease in the metabolism of THC into 11-OH-THC, while estradiol had the opposite effect[368].

Thus, while there is still much to discover, there is enough evidence to support the assertion that there is clear sexual dimorphism in the effects of CBRAs, and that this dimorphism likely stems from both organizational (that is, previous to the influence of gonadal hormones) and hormonal differences between males and females. Moreover, given that in previous animal studies, the depressive-like effects of chronic adolescent CBRA exposure were observed almost exclusively in females[268,276,282,294,308,315,359], in the present work only female animals were employed.

4 – HU-210

Originally synthetized by Mechoulam et al.[372], the highly potent non-selective CBRA, HU-210 ((6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol), is a structural analog of the phytocannabinoid Δ^8-tetrahydrocannabinol[373]. However, despite the structural similarity with this natural cannabinoid (which is less potent than THC at inhibiting AC

activity and cAMP increases[374,374]), HU-210 exhibits remarkably high affinity and efficacy at the CB_1R, being a very potent agonist of this receptor[373,375,376]. Moreover, while no direct data exists as to the half-life of HU-210, it has been predicted – based on the data obtained for its NMDAR-targeting enantiomer HU-211[377] – that this drug may have an exceptionally long half-life. As such, while these characteristics have made HU-210 a widely used pharmacological tool for the exploration of the ECS[373], they have also made it an attractive substance for recreational purposes, with HU-210 having been found in apprehended samples of SC products[378]. Furthermore, while HU-210 shares many pharmacological actions of THC, there is data suggesting that it may interact with other receptors not targeted by the latter drug. For example, while both HU-210 and THC have been shown to potentiate the activation of glycine receptor α1 and α1β subunits[379,380], HU-210 has been demonstrated to also inhibit α2 and α3 subunits of that same receptor[379], as well as to potentiate the activation of the $5\text{-}HT_2$ sub-family of receptors[381].

In the studies that have used HU-210, a pattern of effects similar to those of THC – albeit at a much increased potency – has been widely described. As such, HU-210 was shown to dose-dependently decrease the levels of a number of hormones (such as plasma growth hormone)[373,382], to alter the functioning of the HPA axis – by leading to marked increases in corticotropin releasing factor (CRF), corticosterone (CORT) and adrenocorticotropic hormone (ACTH) levels[373,382] – and to propitiate a decrease in DA levels, as suggested by a decreased L-3,4,-dihydroxyphenylacetic acid (DOPAC)/DA ratio[373,382]. Moreover, at the behavioral level, HU-210 has been demonstrated to induce deleterious effects on locomotor activity, as indicated by marked hipolocomotion, catalepsy[382–384], and a curious repetitive circling behavior[385]. At the level of cognitive function, HU-210 has also been demonstrated have a deleterious impact, as indicated by spatial learning impairments[386,387]. Furthermore, with regard to affective functioning, HU-210 has been shown acutely increase anxiety-like behavior in a number of behavioral assays[383,387,388], as well as to lead to increased vocalization responses to tactile stimulation[385,387], increased aggression[383], and increased grooming (after subchronic administration[388]). Interestingly, the changes reported in anxiety-like behavior seem to be related to the alterations at the in HPA axis functioning, as concomitant injection with a CRF receptor antagonist largely abolished them[383]. In addition, there is some suggestion that HU-210 administration alters the sexual drive of injected rats – either through its hormonal effects, or through its effects on the DAergic system – leading to impaired male sexual behavior, and a decrease in female

receptivity behavior[389], which suggests the possibility that the drug may interfere with the rewarding properties of reproductive activity.

Despite the reported effects of HU-210, and its common use in cannabinoid research, it is rather curious that no study has, thus far, tested the effects of chronic adolescent exposure to this drug on adult affective functioning. This is the case even though, the single study focusing on the effects of adolescent exposure to HU-210 did report several alterations that are suggestive of a prodepressant-like effect, such as decreased hippocampal neurogenesis and increased stress-reactivity[321]. Moreover, this lack of studies becomes even more perplexing when one considers the existence of studies where adult animals chronically administered high-doses of HU-210 not only show increased hippocampal neurogenesis[213], but actual antidepressant-[213,390] and anxiolytic-like[213] responses to drug treatment, both on the day after the end of drug treatment and after a 30-day washout. Furthermore, at least some of these beneficial effects of HU-210 stem from direct or indirect interactions with non-ECS targets, such as the NA system[390].

Thus, in summary, despite the existence of results suggesting a prodepressant effect of HU-210, there is still no conclusive data as to the effects that chronic adolescent exposure to this drug may have on long-term affective behavioral outcomes. Moreover, the existence of adult data pointing towards the opposite effect raises the possibility that HU-210 may either have age-dependent effects, or that it may present characteristics that make it distinct from other previously studied CBRAs, and, as such, worth exploring.

5 – Reasoning, Aim and Organization of the Present Work

As detailed in the previous sections, the effects of chronic adolescent CBRA exposure have been extensively documented in numerous domains and levels of analysis. However, the literature presents a significant gap: specifically, the overwhelming majority of studies were performed using either THC, WIN 55,212-2 or CP 55,940. While these drugs are well characterized, there is some suggestion that their long-term effects may not be comparable – with differences in pharmacology (table 1.1) being a likely culprit. Indeed, while CP 55,940 shares the same relative affinities for CB_1R and CB_2R as THC, it acts as a full, and not partial, agonist at these receptors. Moreover, WIN 55,212-2, also a full agonist at both receptors, is more selective for CB_2R than CB_1R, despite still being classified as a non-selective CBRA.

This overreliance on such a small pool of drugs becomes problematic when one considers two aspects: on the one hand, there is some evidence that different CBRAs may signal through different G-protein subtypes, even though they activate the same receptor[391] (i.e., they present biased agonism), such that it may be the case that the three drugs used may not be capable of evoking the totality of the possible effects of CBR activation. On the other hand, different CBRAs may have different interactions with other, ECS- or non-ECS related, receptors[293], which may lead to different effects.

In this regard it is interesting that the highly potent CBRA, HU-210, has yet to be tested with regards to its effects after chronic adolescent administration. This is the case even though: a) HU-210 is more potent, effective and CB_1R selective (despite also being a non-selective CBRA) than the aforementioned drugs; b) HU-210 has been found in SC products[378], and; c) in the few studies that have used this drug, results have suggested it may have a rather peculiar effect profile: with both prodepressant-[321], and antidepressant-like results having been observed[213,390].

As such, the overarching aims of the present work were to characterize the effects of chronic adolescent exposure to HU-210 with regards to: a) anxiety-like behavior; b) depressive-like behavior and; c) molecular alterations in the ECS. For this, four experiments were performed, each designed to further explore and answer questions derived from the results of the previous experiment.

Because of the results-driven nature of the present work, each experiment constitutes its own separate chapter, with a brief explanation of the rationale behind the experiment and the hypotheses contemplated, the description of the methods used to perform the experiment, the results obtained, and a brief discussion of their possible interpretation. Finally, so as to provide a more global account of the overall project, the last chapter of this work constitutes a general discussion of the results obtained across the four experiments, including both possible explanations for them, as well as a number of ways in which to test the resulting hypotheses, and further understand the results herein obtained.

Chapter 2 – Behavioral Methodologies[c]

The assessment of psychological/psychiatric disorders is, by definition, a difficult endeavor, given the intrinsically subjective nature of these pathologies[394,395]. Since this is a problem even in human research, it is not surprising that in animal research this obstacle is even greater: for one, while humans can, with varying degrees of accuracy, relate their subjective experiences, animals are incapable doing the same, thus eliminating the possibility of assessing several critical and defining features of these disorders – such as the feelings of worthlessness and guilt, or suicidal ideation, in major depressive disorder[314,396]. Furthermore, it is highly questionable whether less developed animals, such as rodents, are even capable of experiencing such complex psychological states[d].

Thus, animal research into psychiatric disorders is deeply reliant on behavioral testing as a tool and method of validation. Indeed, even researchers that focus on the molecular and biological underpinnings of psychiatric disorders, or on the study of causes and/or effects of certain alterations found in humans and replicable in animals, will – at some point in their research programs – need to validate their findings and conclusions with behavioral data, or have it done for them[397]. As an example, in the context of the topic of the present work, had the reports of decreased neurogenesis (one of the most well established biological correlates of depressive disorders[398,399]), in adult animals who underwent chronic adolescent exposure to CBRAs[284,315,321] not included behavioral tests, the findings could only be taken as replications of human correlational data, and not as evidence of causality[397]. That is, it is only by employing behavioral testing that one can determine whether any biological alteration observed is necessary and/or sufficient for the development of a disordered state, and not merely a downstream consequence (or, indeed, an irrelevant correlate) of the real cause of that state[397].

Importantly, the deep necessity for behavioral testing carries with it the implicit assumption that the tests are actually adequate for the job in question. This proposition, however, is not necessarily true (and its truth value varies from test to test), and is contingent on numerous factors, both intrinsic to the test

[c] This chapter is an adapted version of two review papers[392,393] published by the author, during the development of the work here presented.

[d] It is also for these reasons that animal researchers generally use terms such as *depressive-like* or *anxiety-like*, instead of *depressive* or *anxious*, to describe altered behavior, in animal models of these disorders.

itself, such as its validity and reliability, and related to its use, such as the adequate implementation of the test and interpretation of the data obtained.

As such, and given that the current work relies heavily on behavioral testing to achieve its proposed aims, a description and critical appraisal will be made, of each of the behavioral tests used herein. Critically, the tests described and evaluated here are also some of the most widely used behavioral tests in depression and anxiety research.

However, before any evaluation is made, one must first consider the criteria by which this appraisal is commonly done: reliability and validity. The former concept is easily definable, translating the idea that outcomes obtained with one test must be reproducible across experiments and experimenters, for data and conclusions derived from that data to be valid[400]. For this reason, the importance of reliability, is rarely (if ever) contested. Validity, on the other hand, has been a more contentious topic continued of discussion[401]. However, despite this, most animal researchers still use the framework proposed by Willner[402] – who based it on a similar framework used for psychological testing in humans – where three types of validity are contemplated:

Face validity translates the degree of phenomenological similarity between the model and the disease being modeled[402]. That is, the face validity of a model is evaluated by whether the model presents characteristics observed in the disease, without presenting characteristics that are not observed in that same disease. This concern, however, has largely abated in recent years, as researchers have focused less on developing models that capture the entire presentation of any given disorder, opting instead to develop models of specific disease endophenotypes[403,404] (such as the impaired stress-coping behavior that is measured by the mFST).

Construct validity refers to how interpretable and homologous the behavior of the model is, in relation to the disorder being modelled, both theoretically and empirically[402]. Thus, the construct validity of a model relates how well its theoretical account (e.g., its assumptions about disease nature, etiology and biological underpinnings) as well as the empirical observations obtained with it, match those of the disease[402]. This matching between the model and what is being modelled, will define how interpretable any result obtained with the model will be, in relation to the disorder in question.

Lastly, predictive validity refers to the capacity of the model to correctly identify the effects of manipulations (e.g., antidepressant effects of selective serotonin reuptake inhibitors [SSRIs], or the prodepressant effects of chronic stress), without also being vulnerable to false positives or false negatives[402].

In addition, a distinction must be made between methods of inducing psychiatric disorder-like alterations (commonly called animal models), and behavioral tests (also called screening tests). The former are used to study the biology and etiology of psychiatric disorders, as well as the effects that possible treatments may have on those disorders[405]. For this reason, models heavily emphasize construct validity as an important attribute[400]. The methods by which disorder-like alterations can be induced are varied, from environmental (e.g., CMS[406,407]) to genetic manipulations (e.g., animals genetically modified to exhibit a given behavior pattern[408]). Moreover, models can, in and of themselves, include measures by which to assess disordered behavior, or be combined with screening tests for that purpose.

Screening tests, on the other hand, were developed with the primary intent of accurately identifying new possible treatments, or to evaluate the impact on some previous manipulation on a specific dimension of a given disorder[405]. These tests do this through the quantification of a specific, well-defined and easily measurable, behavioral output, that is evoked by the test situation itself[405]. That is, whereas models can be understood to encompass both the dependent and independent variables being studied, screening tests encompass only the former[409]. Furthermore, given the more practical and applied nature of screening tests, they are often evaluated primarily in terms of their reliability and predictive validity[396,409].

1 – Elevated Plus Maze

The EPM[410] is one of the most – if not, indeed, the most – widely used screening tests for the assessment of anxiety-like behavior. Indeed, this popularity is likely the product of the fact that, not only has the EPM been amply validated to work with both rats and mice, but also that it is reliable, as well as easy to use and automate (using video tracking software). In addition, these factors are increased by the fact that the test has been widely shown to be sensitive to the effects of previous manipulations on anxiety-like behavior[411], making it useful not just in pre-clinical pharmaceutical research, but also in basic research settings as well.

Like the SIT and OFT, the EPM is classified as an unconditioned test of anxiety, given that it relies on natural, unconditioned, responses of the animals to some ethological relevant situation for it to work, instead of necessitating the development of a conditioned response to some stimulus[412]. Indeed, this reliance on unconditioned responses has led some researchers to suggest that that tests of unconditioned anxiety may be better analogues of human anxiety[412].

In the case of the EPM, the underlying ethological assumption driving the test, is that it triggers a conflict between two opposing drives. Specifically, it is assumed that by placing the animals in the EPM apparatus – a plus-sign shaped apparatus, that is raised off the ground, where two open arms are perpendicular to two arms enclosed by tall walls on three sides (fig. 2.1) – one is placing the animal in a situation where its natural aversion to open and/or elevated spaces (i.e., the open arms) conflicts with the equally strong drive towards exploration and novelty[413,414]. From this follows that anxiolytic-like manipulations will shift this conflict towards exploration, whereas anxiogenic-like manipulations are expected to do the opposite.

The most widely used EPM protocol consists of a single trial, at the beginning of which the animal is placed in the center of the maze, facing an open arm, being left to freely explore the apparatus for 5 minutes[414]. Many measures can be derived from this test, but the two most common ones are the percentage of time spent, and the total number of entries, in the open arms, which are taken as inverse indexes of anxiety-like behavior[413,414]. Additionally, while some reports derive measures of locomotor activity from this test – such as the number of areas entered over the duration of the trial – these are not adequately validated, and may not be reliable indexes of this parameter[414].

Despite its widespread popularity, the EPM is also the subject of numerous criticisms, concerning both its conceptual underpinnings, and its usefulness for pre-clinical therapeutic research. First, the ethological assumption that underlies the EPM – i.e., that it triggers a conflict between opposing drives – is not an unarguable one. Indeed, one can easily understand the EPM in terms of preference: animals naturally prefer enclosed spaces, and, by putting them in the maze apparatus, one is effectively asking them to choose between a non-aversive – and maybe even rewarding – stimulus (i.e., the closed arms) and an aversive one (i.e., the open arms)[415]. This is even more pressing when one considers that there is very little to actually explore in the EPM: indeed, in other tests relying on the same assumptions as the EPM, such as the OFT,

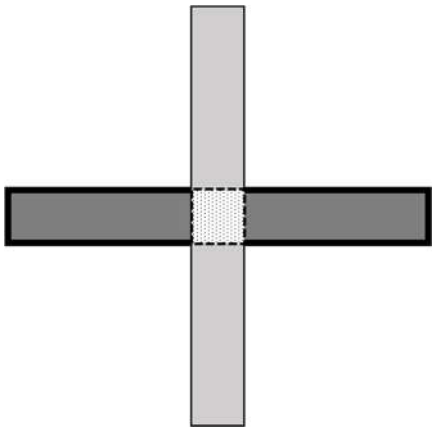

Fig. 2.1 – Schematic representation of the elevated plus maze. The apparatus consists of four arms: two (dark gray) are enclosed on three sides by tall walls (thick black lines), providing a dark space for rodents to hide in, while the other two (light gray) are open. At the beginning of the trial the animal is placed in the virtually defined (dashed lines) center zone (dotted), facing an open arm. Time spent in the open arms is interpreted as being inversely correlated to anxiety.

the time spent in aversive zones is increased if these regions contain objects unknown to the animals[415–417]. Moreover, simple avoidance of an aversive stimulus is, in itself, rewarding[415,418]. Thus, one can ask whether there is *any conflict at all* being evoked by the EPM as, for a conflict to exist, there would have to be a possibility of both negative and positive outcomes resulting from either of the options available[415]. However, this is not the case, as there is little to no possible negative consequence from staying in the closed arms, while the same is not true for the open arms.

Similarly, one can reasonably question whether avoidance of aversive stimuli necessarily implies the existence of an anxiety response[415]. Indeed, there are plenty of quotidian situations where this is not the case, leading to the possibility that EPM-behavior may be interpreted purely in terms of simple avoidance without the necessity of anxiety being considered.

The remaining significant criticism of the EPM focuses on the fact that it seems to be severely lacking in predictive validity, in two ways: firstly, this test has been shown to be incapable of differentiating changes in anxiety-like behavior, from changes in locomotor activity (such as those induced by amphetamine[419]), leading to misleading results[415]. Secondly, the fact that the EPM has been repeatedly shown to not detect the therapeutic effects of drugs known to be anxiolytic[420,421], not only brings into question its adequacy in this regard – and in also detecting anxiogenic manipulations – but also casts doubt into the construct validity of the test: that is, if drugs known to alter anxiety in humans, have no measurable impact on EPM behavior, one can question whether this test is actually measuring anxiety at all[420,421]. In

this line, it has been suggested that tests such as the EPM may only be measuring one of several possible types of anxiety[422] – a proposition yet untested.

2 – Open Field Test

The OFT is one of the oldest behavioral tests still in use, and its popularity rivals that of the EPM. This is likely the case because not only is this test extremely simple to perform and automate, but also because, in addition to providing a measure of anxiety-like behavior[423,424], it also purports to provide a measure of locomotor activity[424]. Indeed, this last reason makes the OFT an extremely common test in depression research, being used to control for possible locomotor alterations, that may bias results from assays such as FST or tail suspension test (TST)[425].

Similarly to the EPM, the ethological underpinnings of the OFT relate to the natural aversion of rodents towards open, brightly lit, spaces, and the conflict between that aversion and the drive towards exploration[415,421]. A marked difference from the EPM, however, comes from the fact that whereas there is an almost complete agreement in the protocol used for that test, the way the OFT is performed varies almost from researcher to researcher[426,427]. Indeed, there is marked variation in terms of the open field (OF) apparatus itself (e.g., shape, color, texture[423,427]), in the environmental conditions in which the test is run (e.g., light level, background noise[423,427]), in trial duration (with some as short as 2 minutes and some taking a full hour[426,427]), in the number of trials performed[426,427], and, critically, in both the way the data is gathered and in how that data is interpreted. Thus, numerous methods can be used to collect data – from highly precise light-beam systems, to video-tracking software, to lines marked on the floor of the apparatus – with measurement precision varying accordingly. Additionally, the choice of data gathering system will necessarily impact what parameters will be measured, further aggravating the fact that the biggest source of heterogeneity in this test concerns the parameters measured and their interpretation. Indeed, numerous parameters can be, and have been, derived from the OFT and – critically – the interpretations derived from the same parameter also frequently vary from researcher to researcher[426,427]. The upshot of this is that what is considered anxiety-like behavior is entirely dependent on the choice of parameters and of interpretation made – resulting in a marked decrease in the reliability of any single result, and in an equally marked

increase in the difficulty comparing results obtained by different groups. Indeed, this is – in addition to the criticism below – one of the main points of contention regarding the OFT.

While ambulation/locomotion is the most commonly used parameter[427], being interpreted either as a measure of rodent exploration[428], arousal/emotionality[429,430], or locomotor activity[423], there are two critical problems with it: for one it leads to a situation where one can reach markedly different interpretations, based on one single outcome (i.e., depending on whether one chooses to see ambulation as indexing exploration or emotionality/arousal, increases in this parameter would imply either anxiolysis or anxiogenesis, respectively). On the other hand, when intending to use the OFT as a simultaneous assessment of anxiety-like behavior and locomotor activity, one faces a critical limitation: decreases or increases in ambulation may be a result of sedation or hyperactivity, respectively, independently of the existence of any alteration at the level of anxiety[424]. Thus, and while an adequate experimental design can be helpful in offsetting this, the usefulness of this parameter as a measure of anxiety-like behavior, is inherently limited by this vulnerability[426].

In addition to ambulation it also common that researchers will quantify ethological parameters (such as the number of grooming episodes), the majority of which lack any appreciable validation[427]. However, due to how commonly used they are, two of these parameters – defecation and rearing – deserve a brief mention: defecation has been an OFT measure ever since the inception of the test[431], and it has been demonstrated to be a reliable index of negative affective states[432,433] (i.e., increased defecation is tied to negative affect). Rearing, however, is a more controversial measure, despite how common its use is. Specifically, while decreases and increases in rearing are commonly interpreted as being indicative of increased[434] or decreased anxiety-like behavior, respectively, there have been reports of increased rearing in situations of increased anxiety[435] – thus bringing into question the validity of this measure[415].

Thus, if one aims to use the OFT as a measure of anxiety-like behavior, one has to focus not just on *how* the animal behaves, but also in *where* it performs that behavior[436]. Specifically, in more recent years, researchers have taken to use an approach whereby the open field is divided (virtually or through line markings; fig. 2.2) into peripheral (PZ), intermediate (IZ) and central zones (CZ). As such, based on the ethological assumption that CZ will be more anxiogenic, due to its open and exposed nature[415,421], the

quantification of time spent and/or distance travelled in each of the three zones, provides measures that are less vulnerable to being biased. Specifically, increased percentage of total time spent and/or distance travelled in PZ[e] or CZ – without concomitant alterations in overall locomotor activity – are interpreted as being indicative of increased or decreased anxiety-like behavior, respectively[424,437].

Irrespective of how popular the OFT is, several authors have highlighted a need for caution when using the test as a measure of anxiety, since it may not be an adequate analogue of pathological anxiety[415,424]: as is the case with the EPM, numerous drugs known to have beneficial effects in the treatment of anxiety disorders have no impact on OFT behavior – indeed, some drugs, such as tricyclic antidepressants (TCAs) actually lead to anxiogenic-like alterations in this test – thus casting into doubt both the predictive and construct validities of this tests[421,424]. This has led some authors to propose that the type of anxiety evoked by tests such as the EPM and the OFT may be more akin to the "everyday" anxiety present under stressful or threatening conditions[424,438], rather than pathological anxiety that characterizes anxiety disorders, which is likely to have different neurobiological substrates[439].

Finally, there is significant criticism of the OFT as a measure of locomotor activity. Specifically, one has to make the important, and often ignored, consideration that, if differences in locomotor activity can bias the measurement of anxiety-like behavior, then the opposite must also be true: that is, any given

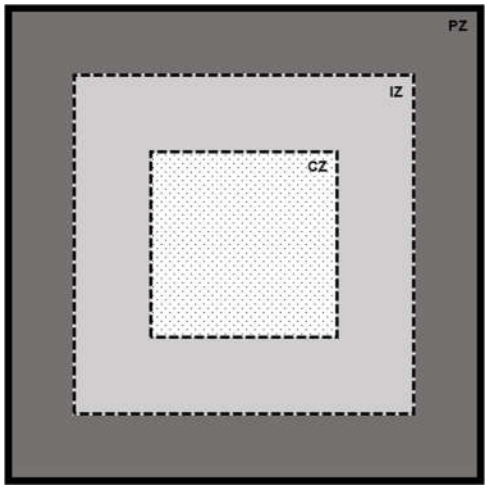

Fig. 2.2 – Schematic representation of the open field test. The open field is virtually divided (dashed lines) into three zones: the peripheral (PZ; dark gray), intermediate (IZ; light gray) and central (CZ; dotted) zones. Increased path length and time spent in the more anxiogenic CZ are taken to be inversely correlated to anxiety. Additional measures can be quantified, such as the number of transitions between zones and the average speed of the animal.

[e] It is common for the proclivity of rodents for walking close to the walls of the open field to be described as "wall-walking" or "thigmotaxis". This behavior is similar in nature to that observed in the EPM, whereby rodents prefer darker, less exposed areas.

pattern of locomotor activity may be as much affected by the purported anxiogenic nature of the testing situation, as by any actual locomotor activity altering effects of the manipulation in study[426,440]. This is even more relevant when the OFT consists of a single, short-duration, session, where anxiety is presumed to be at its maximum. Furthermore, there seems to be little to no appreciable correlation between OFT and home-cage measurements of locomotor activity[441], suggesting that either a) anxiety is exerting an unappreciated biasing effect on locomotion in the OFT or; b) the OFT is simply inadequate to assess this parameter. Indeed, this has led Stanford to suggest that, despite the heightened logistical requirements, locomotor activity can only be reliably assessed through home-cage-derived measures[441].

3 – Social Interaction Test

The SIT was originally developed by File and Hyde[442], with the purpose of using ethological valid sources of anxiety (e.g., light intensity) and naturally occurring behaviors (social interaction) to assess anxiety-like behavior[443,444]. Curiously, however, subsequent research found that SIT results do not correlate to those of other anxiety tests[311], suggesting the possibility that it may be measuring a different construct altogether, such as social anxiety. Indeed, there is also a legitimate argument for the SIT as straddling the line between the assessment of anxiety and of reward-functioning – given that social interaction is a highly rewarding stimulus for rodents[310] – which would explain the lack of correlation to other tests of anxiety.

In the original, and most used version of the SIT protocol, animals are individually habituated to the testing apparatus (commonly the open field), for 10-minutes in the 2 days preceding the testing. On the third day, animals are again placed in the apparatus for the same duration, with the sole difference being the presence of a previously unknown social partner, of equal sex, age, and weight (so as to avoid confounds relating sexual or dominance drives)[442]. Thus, the primary measure of interest is the time that the test animal chooses to spend in active social interaction – defined as "sniffing, following, grooming, kicking, mounting, jumping on, wrestling and boxing with, crawling under/over the partner"[442] – based on the premise that, when exposed to anxiogenic stimuli, rodents will spend less time interacting with novel social partners[442]. As such, in the SIT, increased anxiety-like behavior is signed by a decrease in the time

spent in social interaction (in the absence of concomitant decreases in locomotor activity), whereas the opposite is true for decreases in anxiety-like behavior (assuming no concomitant increase in locomotor activity)[443,444]. Furthermore, while it is common that researchers will also quantify passive social interaction – defined as the animals "sitting or lying with their bodies in contact, but without interacting with each other"[443] – as well as a number of other ethological parameters, such as grooming episodes[445], these measures lack validation and may not be reliable.

Several criticisms can be leveled at the SIT. First, like the EPM and the OFT, there is an inherent vulnerability of test measures to being biased by possible locomotor activity-related alterations[444]. However, one can somewhat try to circumvent this limitation by quantifying locomotor activity in both in habituation and test sessions, despite the limitations that this type of assessment may have (see section 2). Secondly, the rewarding nature of social interaction may be a point of entry for a further confound, since manipulations that impact reward function may conceivably be mistaken for alterations in anxiety[309,310]. However, it seems that this possibility has yet to be tested. Thirdly, there is data suggesting that male and female rats may respond differently to this test, with females showing reduced effects of arena familiarity on time spent in social interaction[446]. Finally, as is the case with the EPM and OFT, it seems to be the case that the SIT has limited predictive validity, given that numerous anxiolytic drugs do not affect test performance[415,421,444,447]. Moreover, this lack of predictive validity also implies a somewhat questionable construct validity – although, in the case of this test, given that there is some question has to what the SIT is actually assessing, it is not as pressing a concern.

4 – Modified Forced Swim Test

The mFST[448] is one of the most used screening tests in depression research. This test was derived from the previous – and still commonly used – FST, designed by Porsolt et al[449]. The popularity of both tests derives from their ease of use, the low time and resource investment required to perform them[450], as well as from the fact that they have been demonstrated to be sensitive to the effects of prodepressant manipulations[451].

The FST is based on the observation that rodents, when placed in an uncovered water-filled cylinder, from which they cannot escape, will initially try to do so, but will – after a short amount of time – stop

trying to actively escape, adopting an immobile behavior, keeping only their head above water[450]. This behavior was originally interpreted as being a form of "behavioral despair"[452], similar in nature to the learned helplessness phenomenon described by Seligman[453]. In more recent years, however, this interpretation has come to be largely replaced by an understanding of the behavior as representing a shift from an active, energy-expensive, coping strategy, to a passive one, in an effort to conserve energy[454–456]. While this interpretation is not as directly related to the conception of the FST as an assay of depressive-like behavior, as that of behavioral despair, it is nonetheless congruent with the endophenotypic approach described in the first section of this chapter. Indeed, altered stress coping is one of the paramount symptoms of depressive disorders[457], and it may be argued that the FST (and, consequently, the mFST) is a test of this particular aspect. Moreover, notwithstanding the interpretation chosen, it is expected that antidepressant treatments will decrease immobility – defined as the animals "floating passively in the water making only those movements necessary to keep their heads above water"[452] – and that prodepressant treatments will increase it[450].

The driving force behind the development of the mFST was a limitation of the original test: specifically, the FST was shown to have limited predictive validity in identifying SSRIs[396,448,458,459] – the first line of treatment for depressive disorders[460] – as being antidepressants. This led Detke et al.[448], to introduce several changes to the protocol, while keeping some aspects in common between the two tests: whereas, like the FST, the mFST consists of two sessions (lasting 15 and 5 minutes, respectively) on consecutive days[450] – so as to allow for the development of stable behavioral patterns in the second swim[450] – in the mFST water depth was increased from 15-18 to 30 cm, so as to avoid animals from being able to touch the bottom of the cylinder and using their tails to stabilize themselves, an alteration that led to decreased levels of immobile behavior[448]. Moreover, whereas in the FST the only behavior quantified was immobility, in the mFST a distinction is made between two types of active behavior, allowing for the correct identification of SSRIs as antidepressant: climbing and swimming[448]. The former is defined as the animal making "active movements with its forepaws in and out of the water, usually directed against the walls"[448], while the latter is defined as the animal making "active swimming motions, more than necessary to merely maintain its head above water"[448] (fig. 2.3). Critically, while immobility is expected to be decreased by all

antidepressant treatments, the active behaviors are differentially affected by pharmacological manipulations, such that drugs targeting 5-HT activity (such as SSRIs) will increase swimming behavior, whereas therapeutic drugs targeting NA signaling will selectively increase climbing behavior[461]. Finally, whereas in the FST scoring is done in a continuous, cumulative manner (i.e., the scorer will count the number of seconds spent in immobility during the entirety of the trial), for the mFST a time-sampling technique was introduced, whereby scoring is performed in 5 second intervals, with each interval being classified in terms of the predominant behavior displayed during that interval[448]. It should, however, be noted, that it is still common[462] that the continuous cumulative manner of scoring be used in the mFST.

The primary criticism made of both the FST and mFST concerns the susceptibility of these assays to being biased by alterations at the level of locomotor activity. Indeed, when testing pharmacological treatments in these assays, the majority of false positives (e.g., amphetamines) and false negatives (e.g., benzodiazepines) result from the locomotor hyper and hypoactivity induced by these drugs, respectively[425,450,459]. Thus, when using the FST/mFST it is imperative that one obtain some measure of locomotor activity – something that is commonly done via the OFT. Another concern, less relevant context of the present work, focuses on the fact that neither version of the test accurately replicates the temporal dynamics of antidepressant treatment effects: whereas, in humans, antidepressant drugs have to be chronically administered (2-4 weeks) for effects to be appreciable, in these behavioral assays acute administration is enough for effects to be detectable[450]. However, while this is an important criticism, it has been somewhat curtailed by findings that not only is there rapid improvement in some symptoms of

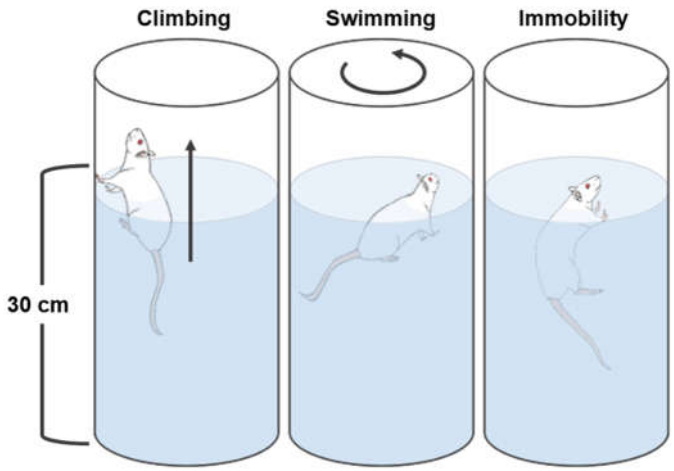

Fig. 2.3 – Schematic representation of the modified forced swim test. Animals are placed into a cylinder filled with water to a depth of 30 centimeters. During the trial three types of behavior are quantified: climbing, swimming and immobility (see definitions in text), with increased immobility being interpreted as being indicative of increased depressive-like behavior.

depression, in humans being treated with antidepressants[463], but also that the acute effectiveness of drugs in these tests is only present when large doses are administered, with lower doses requiring prolonged administration[450].

5 – Sucrose Preference Test

Marked reductions in interest or pleasure, referred to as anhedonia, are one of the key diagnostic criteria for depressive disorder[314]. Thus, tests that can reliably assess the effects of manipulations on this parameter are critically important for depression research[309]. However, testing reward functioning in animals is not as easy as testing the response to acute inescapable stress: when testing the effectiveness of antidepressant manipulations on reward functioning, researchers have to first induce a deficit in this domain by some means[464], so that a beneficial effect of treatment may then be detected. Indeed, the SPT was developed in the context of such a prodepressant manipulation – the CMS paradigm[465,466] – based on the finding that animals previously exposed to mild but inescapable stress, would, overtime, develop decreases in both intake of, and/or preference for, a sucrose solution (generally 0.5-2%), when given a choice between two bottles, one of which contained only tap water[406,467]. Moreover, in contrast with the immediate effects of acute antidepressant administration in the mFST, in the SPT a beneficial treatment effect is only observed after chronic treatment – more faithfully replicating the temporal dynamics of antidepressant drug treatment.

However, despite co-originating with the CMS paradigm, the SPT has since been shown to work with other methods of inducing deficits in reward functioning. Furthermore, from its development onwards, the SPT has become almost as heterogeneous as the OFT, in terms of the ways it is performed. Indeed, there is variability with regards to where the test is performed (i.e., home-cage[466] vs specific housing[294]), in the moment of application (during[466] vs. after the induction protocol[294]), in its duration (e.g., 1[468] to 72 hours[294]), in the number of trials (e.g., weekly[466] vs. single testing[294]), in whether testing is performed with animals isolated[466] or not[469], in whether a period of habituation to sucrose exists[466] before testing or not[294], in whether animals undergo food/water deprivation before testing[466] or not[294], in whether calorie-containing sucrose[294] or calorie-free saccharin[470] is used for solutions, in the concentration of the

sweetened solutions[294,466,471], and in whether one measures absolute intake[472], relative preference[473] or both[474]. Evidently, as is the case with the OFT, this is a significant point of criticism for this test, as it hinders the determination of exactly how valid and reliable the SPT, and any results obtained with it, actually are.

Another possible criticism pertains to the fact that the SPT cannot adequately distinguish if altered behavior stems from impaired reward sensitivity (i.e., an inability to feel pleasure) or, alternatively, from altered reward valuation (i.e., low relevance attributed to pleasurable stimuli, despite intact reward sensitivity)[475]. Indeed, reward functioning is a multidimensional concept[309], that encompasses not just the consummatory component tested by the SPT, but also anticipatory, motivational and learning components, all likely having different neurobiological substrates, which may be differentially affected[475].

Finally, the face validity of the SPT has also been questioned in two ways: first, notwithstanding the relevance given to anhedonia as a diagnostic criterion of depressive disorders, there is data suggesting that this symptom is only moderately associated with disorder severity[476]. On the other hand, when humans suffering from depressive disorders have been tested in situations similar to the SPT, they did not show decreased preference for the sweet solutions[477,478].

6 – Marble Burying Test

In recent years the marble burying test (MBT[479]) has been gradually becoming one of the more widely used behavioral tests. This is all the more remarkable, when one considers how much discussion there is over the nature of this test, and what it is actually measuring. Nevertheless, it is likely that this rise in the adoption of the MBT is the consequence of two factors: for one, the MBT is extremely easy to quickly perform in large numbers of animals, in a relatively short amount of time, only requiring cheap and easily obtainable materials. Secondly, despite the discussion over the construct in study, the MBT has one of the best – if not the best – predictive validities of any screening tests for anxiety targeting drugs. Indeed, whereas the EPM or OFT are not sensitive to the anxiolytic effects of antidepressant drugs such as SSRIs or TCAs, these effects are reliably detected by the MBT[420,421].

The MBT is a more ethically acceptable[480,481] version of the older shock-probe test[482]. In this latter test animals are individually placed in a cage where a small electrified probe is present, that will mildly shock

the animal when it touches it[482]. This shock is theorized to trigger a natural rodent defensive response, whereby the animal will defensively push cage bedding towards the probe, effectively burying it[482]. Indeed, in natural settings, rodents are known to displace soil towards predators entering their burrows[481]. Critically, this burying response is expected to be decreased by anxiolytic drugs, and increased by anxiogenic drugs[481].

The MBT does away with the use of a shock-probe (hence being more ethically acceptable[481]), ostensively using novelty as the aversive stimulus,[483] instead: in this test, animals[f] are individually placed in a cage with lightly tamped bedding, on top of which a number of objects – generally glass marbles – are evenly distributed[481]. After the end of the 30-minute trial, animals are removed, and the number of marbles buried – that is, with at least two thirds of their volume covered (fig. 2.4) – is counted[481]. As in the case of the shock-probe test, anxiolytic treatment is expected to reduce the number of marbles buried, with anxiogenic treatment being expected to have the opposite effect.

Despite the high predictive validity of the MBT[420,421], there is significant discussion over what the test is actually measuring[481,483–485]. The idea that marble burying behavior is the product of novelty-induced anxiety, would make the MBT a test of unconditioned anxiety, like the EPM, OFT or the SIT. However, there is significant indication that this may not be an accurate interpretation: for one, if novelty were the anxiety-inducing stimulus, it would be expected that previous habituation to the marbles would lead to a decrease in the number of buried marbles – something that is not observed[483,485]. Secondly, it would be reasonable to expect that, if marbles were an aversive stimulus to animals, they would avoid them if given the chance. However, this is not the case, as animals seemingly make no special effort to avoid the marbles, even when given ample opportunity to do so[483,485]. Thirdly, while it would also be expectable that animals would only bury stimuli that have aversive properties, the fact is that even stimuli with which animals are amply familiarized, and which are completely devoid of aversive properties – such as food pellets – are readily buried[485,486] (although to a slightly lesser extent[485]). Finally, it would be expectable that performance in this test would correlate to performance in other tests of unconditioned anxiety, however this is not the case, as MBT outcomes do not correlate to either EPM, OFT or LDBT scores[480,485].

[f] It should be noted that while the MBT is more commonly performed with mice, there is ample evidence that rats also share this type of behavior. Indeed, there is some suggestion that defensive burying behavior is more directed, and deliberate, in rats than it is in mice.

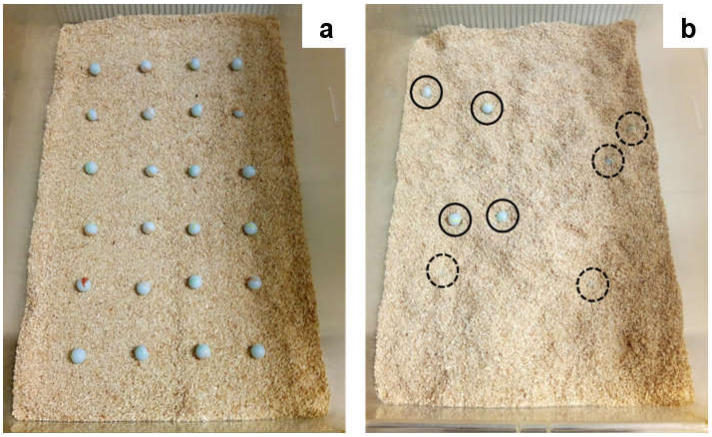

Fig. 2.4 – Representative pictures of the marble burying test. Animals are individually placed into a cage filled with 24, evenly distributed, marbles **(a)**. At the conclusion of the 30-minute trial **(b)**, animals are removed, and the number of marbles buried up to two thirds of their volume (dashed circles) is scored. Full circles identify marbles left unburied.

In combination, this has led some researchers to suggest that the MBT might not be a test of anxiety, but that it may, instead, be a test of repetitive/compulsive behavior[485] – such as that observed in obsessive-compulsive disorder (OCD). While this would, *prima facie*, fit with the observations described above, there are some significant problems with this assertion: for one, that it would be normal, and not disordered, animals showing this type of pathological behavior[481,487], makes little sense. Indeed, whereas anxiety has adaptive functions in the wild, it is difficult to come up with an adaptive explanation for this type of (seemingly purposeless) behavior, especially when directed at innocuous stimuli with which the animals have ample experience. Secondly, there is some evidence that some habituation occurs, if several trials are performed on the same day[485] – which would not be expected to exist for a compulsive behavior. Moreover, if compulsion was the underlying drive for burying behavior, it would be reasonable to assume that, when given the choice between an environment containing marbles and one without them, animals would choose the former. However, previous studies have shown this to not be the case[483,485]. In addition, the fact that the disposition of the marbles reliably alters how many the animals will bury[483], does not fit with the idea of a compulsion-driven behavior. Thirdly, that MBT behavior does not correlate to EPM, OFT or LDBT performance, while unexpected, should not be seen as indication that this test does not index anxiety: indeed, not only does the SIT also not correlate to other tests of unconditioned anxiety[311], but also there is both the possibility that these other tests may not be measuring pathological anxiety, and/or that there may be different dimensions of anxiety, one of which is uniquely detected by the MBT[422,424,438]. Finally, there is a question relating to the fact that, while SSRIs, TCAs and other antidepressant drugs are effective in

the treatment of OCD, benzodiazepines are not, but nonetheless reliably decrease marble burying[483] – thus supporting a view of the MBT as a test of anxiety.

Given that neither interpretation of the MBT fully fits with the observations obtained with it, a third possible understanding of the test has been proposed. Specifically, authors have increasingly supported the view that marble burying may be merely an accidental byproduct of another, more adaptive, natural rodent behavior – that of digging and burrowing in a new environment[481,487–489]. Indeed, even in the absence of any object, rodents will readily exhibit increased digging activity when placed in cages with new substrate, likely as a product of both exploration and a drive towards building a burrow in which to hide[487]. In line with this, and despite there being some dissociation between digging behavior and the number of marbles buried[485], there is a strong correlation between the two[490,491]. Furthermore, the fact that the number of marbles buried, as well as the way in which that burying is done, is much more affected by the density of the bedding substrate[491], than by any other factor studied to date, supports this line of reasoning. Thus, Njung'e & Handley proposed that marble burying may be better understood as a "correlational model for detection of anxiolytics rather than an isomorphic model of anxiety"[483]. Importantly, however, this understanding should be widened to also include anxiety-inducing manipulations, as evidence suggests that this test is also sensitive to these[492].

Thus, in conclusion, the MBT is still very poorly understood in with regards to what it is actually measuring, with all three interpretations being still commonly used. In the present work, however, the MBT will be interpreted in terms of anxiety-like behavior, given that not only it may be a more sensitive measure of this parameter than either the EPM or the OFT, but also that the evidence with pharmacological manipulations[420,421] – which are the type of manipulation used in this work – supports this interpretation of the test.

Chapter 3 - Experiment 1

1 – Rationale

As stated in the introduction, no study to date has adequately tested the long-term effects of chronic adolescent HU-210 exposure on affective functioning. Indeed, the single report studying this type of exposure to this drug[321], found it to lead to gender-dependent changes whereby, increased corticosterone responses to stress, as well as decreased hippocampal neurogenesis were found exclusively in male animals previously treated with HU-210[321]. Importantly, while neither of these findings can be directly interpreted in terms of depressive-like effects, both are suggestive of a possible deleterious effect of HU-210 on affective functioning, as both have been reported as correlates of depressive-like phenotypes[399,493–495]. However, the fact that these effects were not found in female animals is somewhat in contradiction with the previous literature, in which depressive-like effects are predominantly found in female animals[268,269,276,280,282,294,308,315]. These contradictory findings, in combination with the fact that studies with adult animals have reported chronic exposure to high doses (100 µg/kg) HU-210 as having antidepressant-like effects in the mFST – both 24 hours[390], as well as 30-days after the end of drug exposure[213] – raise the possibility that this drug may be qualitatively different from other CBRAs, with respect to its long-term consequences.

As such, the present experiment was designed to assess whether chronic adolescent exposure to HU-210 leads to any alteration at the level of adult affective-related behavior. Finding that adult HU-210-treated animals present impaired performance, in tests of depressive-like behavior (i.e., mFST and SPT) would support the notion that chronic adolescent exposure to CBRAs persistently alters affective functioning, as well as that the antidepressant-like effects observed in adult animals chronically treated with HU-210 are likely an artefact of different testing ages[496]. Conversely, finding a beneficial effect of HU-210 treatment on adult behavioral performance, in line with the aforementioned adult antidepressant effects, would suggest that HU-210 is somehow different from other previously studied CBRAs, with regards to its impact on affective functioning.

2 – Methods

2.1 – Animals and Ethical Approval

Twenty-five female Wistar rats, aged 21 days (PND 21) at the time of arrival, were ordered from Charles River Laboratories (Lyon, France) and housed in groups of five, in clear plastic cages filled with corn cob shavings as bedding material. Animals were kept on a 12-hour light-dark cycle (lights on from 6:00 to 18:00), with housing facility temperature (22ºC) and humidity (70%) maintained at stable levels. Animals were given ad libitum access to both food and water for the entirety of the stay at the animal facility, and were provided several environmental enrichments, such as aspen wood blocks, transparent red acrylic rat tunnels and cardboard tubes. A period of at least three days of acclimatization was allowed before any experimental procedure was performed, and animals were monitored daily for physical and behavioral signs of distress and/or suffering.

All experiments took place during the light phase of the cycle, and were performed in conformity with European Community Guidelines (Directive 2010/63/UE), and with the approval of the Committee for Ethics in Animal Research of the Faculty of Medicine of the University of Lisbon, as well as of the Portuguese Competent Authority for Animal Welfare.

2.2 – Drugs

HU-210 ((6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; Tocris Bioscience, Bristol, UK) was suspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) resulting in a stock solution at 1mM concentration. Aliquots were prepared and stored at -20ºC until the day of use. From this stock solution further dilutions were made each day, in 0.9% saline, to reach adequate volume.

2.3 – Drug Administration

At the time of arrival animals were randomly assigned to be treated with either HU-210 (n=10), vehicle solution (VEH; n=10), or to serve as stimulus animals during the SIT (n=5).

Drug administration (fig. 3.1) was performed according to a slightly modified version of the protocol used by Lee et al.[321]: Daily intraperitoneal (i.p.) injections of HU-210 were administered five days per

week for three weeks, in an escalating dosing schedule (PND 35-39: 25µg/kg; PND 42-46: 50µg/kg; PND 49-53: 100µg/kg), at a volume of 1 ml/kg. This administration schedule was designed so as to both reduce the likelihood of animals developing tolerance to drug effects (shown to develop more rapidly in younger rats[497]), and to mimic the pattern of increasing use reported by humans[498]. Intermission periods of two days between dosing steps were introduced, to both further diminish the likelihood of tolerance development, and mimic the pattern of frequent, voluntary and involuntary periods of sobriety. To minimize animal stress and facilitate injection procedures, animals were handled for at least three days before the first injection took place.

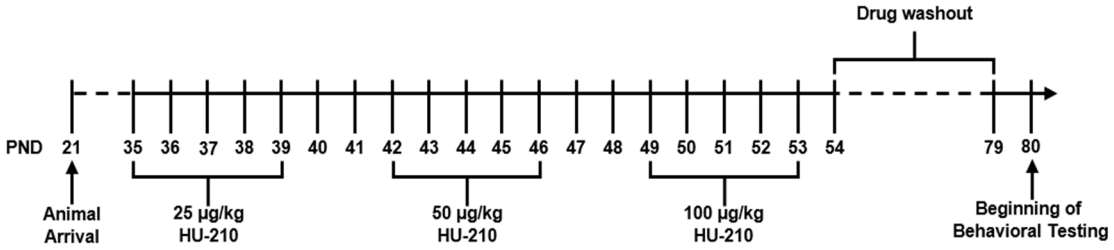

Fig. 3.1 – Timeline of drug administration.

2.4 – Animal Body Weight

Animal body weight was monitored for the entirety of the experiment. During the drug administration period (PND 35-53) animals were weighed daily. To avoid excessively disturbing them, animals were weighed weekly, for the duration of the washout period (PND 54-79). Finally, during the testing period (PND 80-91) animals were weighed at the start of each behavioral testing day.

To calculate weight changes, the starting body weight of each animal at PND 35 was subtracted to its body weight at each time-point[294], with the resulting values (expressed in grams) being used to compare the effects of treatment across groups, at the various stages of the experiment.

2.5 – Behavioral Testing

After drug administration ended (PND 53), animals were given a 27-day drug washout period, during which they were only disturbed for weekly weighings, and periodic cage cleanings. To reduce animal stress, and its possible confounding effect on behavioral performance, rats were individually handled for at least five minutes, on the five days preceding the first test (PND 75-79). Furthermore, to diminish the

likelihood of stress induced by one test influencing the results of the following, two-day rest periods were introduced wherever possible. A chronogram of behavioral testing procedures is described in fig. 3.2.

On testing days, animals were allowed to acclimatize to the testing room for at least 30 minutes, with light and temperature conditions kept stable throughout the testing period. All testing occurred from 8:00 to 18:00, and animals were always tested in the same order, alternating between groups every five animals (i.e. after all animals in one cage had been tested).

In the EPM and OFT, animal behavior was recorded and analyzed using the SMART®2.5 video-tracking software (Panlab, Harvard Apparatus, Barcelona, Spain), with the dorsum of the animals as the reference point used for tracking. On the other hand, the SIT and mFST were recorded using a Brio 4K Pro camera (Logitech, Lausanne, Switzerland), and the resulting videos were analyzed posteriorly, using the Solomon Coder beta version 17.03.22 (András Péter, Milan, Italy) behavior coding software.

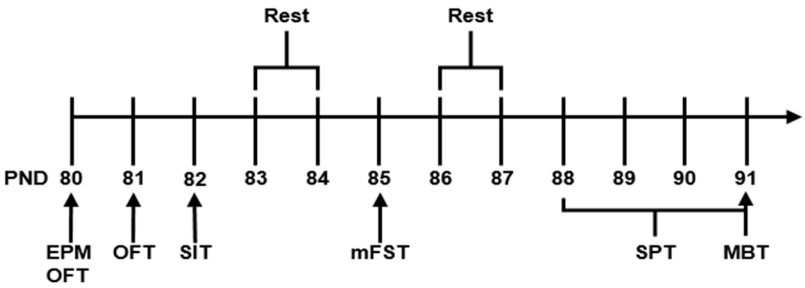

Fig. 3.2 – Chronogram of behavioral experiments performed. PND, post-natal day; EPM, elevated plus maze; OFT, open field test; SIT, social interaction test; mFST, modified forced swim test; SPT, sucrose preference test; MBT, marble burying test;

2.5.1 – Elevated Plus Maze

The day after the end of drug washout (PND 80) animals were tested for anxiety-like behavior in the EPM[413]. The testing apparatus consisted of a plus sign shaped platform, elevated 50 cm above the floor, composed by two arms with no walls (open arms; 10 x 50 cm), perpendicular to two arms surrounded by walls on three sides (closed arms; 10 x 50 x 30 cm). At the start of the trial animals were placed in the intersection between the four arms (center zone), facing an open arm, and were allowed to explore the entirety of the maze for 5 minutes. The apparatus was virtually divided into three zones corresponding to the open and closed arms, as well as the center zone. After trial conclusion, animals were returned to their home cages, and the test apparatus was cleaned with a 30% ethanol solution, to erase olfactory clues.

Two measures were derived from this test: time spent in the open arms (expressed as a percentage of total trial duration) and the number entries in the open arms, both of which were taken as inverse indexes of anxiety-like behavior.

2.5.2 – Open Field Test

Two OFT trials were performed, as part of the habituation period for the SIT, with different measures being derived from each session.

The first trial took place on PND 80, after all animals had been exposed to the EPM. Because first exposure to the OF is likely to be more anxiogenic and, thus, a less reliable measure of locomotor activity (see chapter 2), this session was scored for parameters relating to anxiety. Specifically, permanence time and distance traveled in CZ (expressed as percentages of total time, and distance traveled, respectively) were taken as inverse indexes of anxiety.

The second trial took place on PND 81, and was assessed for parameters related to locomotor activity. Specifically, average velocity (expressed as cm/s) and total distance traveled (expressed as cm), were taken as indexes of this parameter.

Testing apparatus consisted of an empty square box (60 x 60 x 40 cm), virtually divided into three concentric square zones, respectively designated PZ, IZ, and CZ. In both trials animals were individually placed into the center of the OF, and allowed to freely explore for 10 minutes, after which they were returned to their home cage. The test apparatus was cleaned with a 30% ethanol solution, between each animal, to erase olfactory clues.

2.5.3 – Social Interaction Test

The SIT[444] consists of two phases, spread over three consecutive days. The habituation phase consists of two 10-minute sessions, over the first two days, during which animals are allowed to explore the testing apparatus (in this case the OF) by themselves. This period coincided with the aforementioned OFT trials, and was also performed for the five stimulus animals. The test phase occurred on the third day (PND 82), and also had a duration of 10 minutes. However, in the test session, when subjects were placed in the OF, another, sex- and weight-matched, rat (the stimulus rat) was already present.

The primary measure derived from the SIT is the time spent in active social interaction, being expressed as the amount of time (expressed in seconds) that the test rat spent "sniffing, following, grooming, kicking, mounting, jumping on, wrestling and boxing with, crawling under/over the partner"[444].

To avoid the possible confounding effects of fatigue, each of the five stimulus animals was never used in two consecutive trials and as much as time as possible was given between each trial using each individual stimulus rat.

2.5.4 – Modified Forced Swim Test

The mFST was performed as previously described[276]. Briefly, a single 15-minute session was performed on PND 85, during which animals were individually placed into a glass cylinder (20 cm diameter), filled with water maintained at a temperature of 23-25 ºC, to a depth of 30 cm. After each trial ended, animals were removed from the water, dried with a warm towel, and placed under a heating lamp for a period of at least 15 minutes. Additionally, to avoid possible confounding effects, resulting from scent clues, water was replaced between animals.

Three behaviors were scored during the duration of the trial: a) *climbing,* defined as the rat making "active movements with it forepaws in and out of water, usually directed against the walls[448]"; b) *swimming*, defined as the rat making "active swimming motions, more than necessary to merely maintain its head above water[448]", which included sporadic bouts of diving and; c) *immobility,* defined as the rat making "only the movements necessary to keep its head above water[448]". Scoring was done by quantifying the total time spent in each behavior (expressed in seconds) during the trial.

2.5.5 – Sucrose Preference Test

The SPT was performed as previously described[294]. Briefly, from PNDs 88 to 91, animals were individually housed, and allowed ad libitum access to food, as well as to two drinking bottles: one containing tap water, and the other containing a 2% sucrose solution. Bottles were weighed at the start of the test, and every 24 hours afterwards, for the duration of the testing period. To avoid preference or learning effects, the position of the bottles was changed daily, after weighing.

Several parameters were derived from this test: for each time-point (24, 48, and 72h) sucrose intake (normalized for body weight at the start of the test [PND 88]; expressed in milliliters per gram of body

weight) and sucrose preference (expressed as the percentage of sucrose solution consumed relative to total fluid intake[g], whereby values over 50% indicate preference for sucrose, and values below 50% indicate preference for water) were calculated. Additionally, average daily sucrose intake (expressed in milliliters/gram of body weight) and sucrose preference[h], over the testing period, were also calculated.

2.5.6 – Marble Burying Test

The MBT was performed on PND 91, after the final SPT measurements had been obtained. The test was performed as previously described[499], with some slight modifications: briefly, five full size cages were filled with at least 5cm of lightly tamped bedding material, providing a flat surface. Six evenly spaced rows, of four light blue ceramic marbles, were placed on top of the bedding, totaling 24 marbles per cage.

Each animal was placed in a cage, and left undisturbed for 30 minutes. After that time animals were removed and returned to their original group-housing conditions. Between trials visible fecal boli were removed, bedding was again tamped flat, and marbles were cleaned with a 30% ethanol solution. The dependent variable of interest was the number of marbles buried, which was taken as an index of anxiety-like behavior. Marbles were considered buried if more than two thirds of their volume was covered.

2.6 – Statistical Analysis

All comparisons were made between treatment groups, with statistical significance level (α) established at .05. Outliers were detected using the method outlined by Tukey[500]: observations found to be outside of the interval defined by the first quartile (Q1) - 1.5 interquartile range (IQR) and the third quartile (Q3) + 1.5 IQR, for that group, were considered outliers and subsequently removed from analysis. After outlier removal, and before any analysis was conducted, data from each group was tested for normality, using the Shapiro-Wilk normality test. Where no violations of normality were found ($p > .05$), data was analyzed through two-tailed unpaired Student's *t* tests, with the Holm-Sidak correction for multiple comparisons when appropriate, with data expressed as means ± standard error of mean (SEM). Where the assumption of normality was not met, data were analyzed with Mann-Whitney U test, with data being expressed as

[g] $Sucrose\ Preference = \frac{Sucrose\ Intake}{Total\ Fluid\ Intake} * 100$

[h] $Averaged\ Sucrose\ Preference = \frac{(Sucrose\ Intake\ Day\ 1 + Day\ 2 + Day\ 3)}{(Fluid\ Intake\ Day\ 1 + Day\ 2 + Day\ 3)} * 100$

medians and IQR, or range (minimum and maximum values), wherever appropriate. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

3 – Results

3.1 – Animal Body Weight

To assess the effect of chronic adolescent exposure to CBRAs on body weight, the weight changes were measured relative to baseline weight at PND 35. At this initial measurement moment, a significant difference in absolute weighr was found between groups, such that HU-210-assigned animals were found to weight significantly more than their VEH-assigned counterparts (VEH = 134 ± 2.34, HU-210 = 143 ± 2.23; $t(18) = 2.63$, $p = .017$; fig. 3.3).

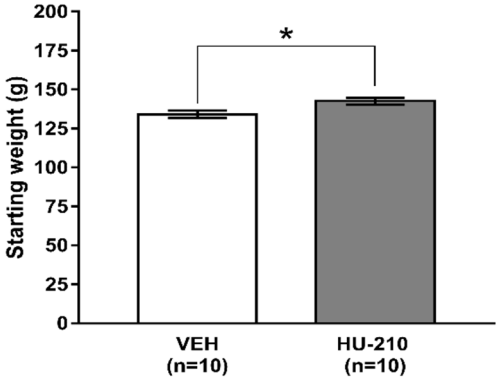

Fig. 3.3 – Animal weights at the start of the experiment (PND 35). HU-210-treated animals weighed significantly more than controls. Data are expressed as mean ± SEM (n=10); * $p < .05$, unpaired Student's *t*-test.

On PND 36, and in every time-point during the drug administration period, HU-210-treated animals showed statistically significant decreases in weight-gain, in comparison to controls (all $p \leq .001$). These differences in weight-gain persisted during part of the drug washout period, being significant at PND 59 ($p \leq .001$) and PND 66 ($p < .05$). However, from PND 73 onwards, the effect of HU-210 treatment ceased to be significant, with both groups showing similar weight-gain for the remainder of the experiment (fig. 3.4).

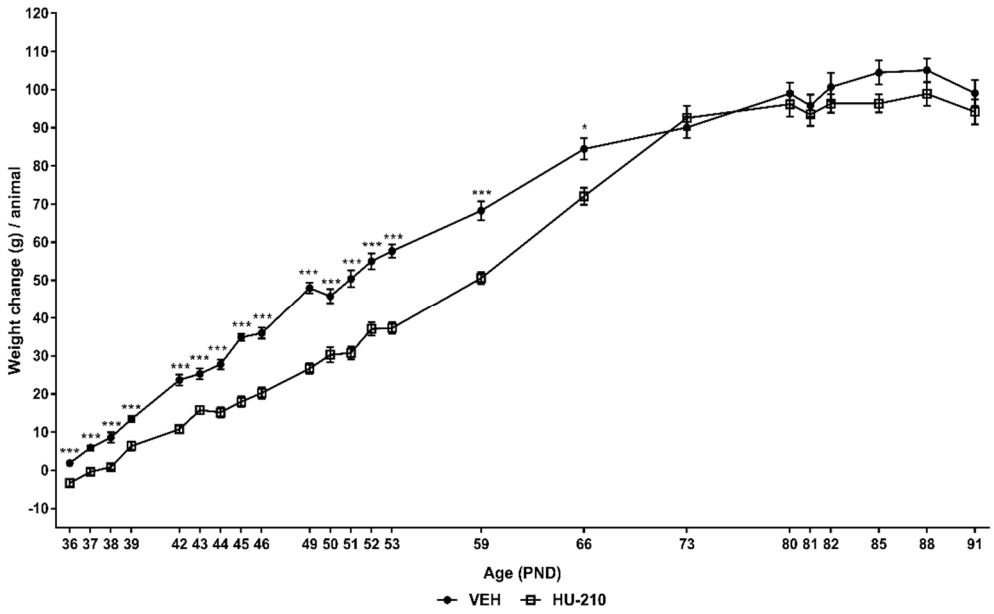

Fig. 3.4 – Change in animal weight relative to PND 35 over the course of the experiment. HU-210 treatment significantly reduced the amount of weight animals gained during the drug administration period (PND 35-53), and for 15 days after its end (PND 63). This effect did not, however, persist for the remainder of the experiment. Data are expressed as mean ± SEM (n=10); * $p < .05$, *** $p \leq .001$, unpaired Student's *t*-test with Holm-Sidak correction.

3.2 – Behavioral Testing

3.2.1 – Elevated Plus Maze

To assess the effects of chronic exposure to HU-210 during the adolescent period, on anxiety-like behavior, the percentage of time spent in, and the number of entries in the open arms of the EPM were compared between groups. No differences were found for either the first (VEH = 25.7 ± 5.29, HU-210 = 32.5 ± 5.92; $t(18) = 0.852$, $p = .406$; fig. 3.5a) or second (VEH = 9.5, range: 4 – 16, HU-210 = 8.5, range: 0 – 15; $U = 48$, $p = .896$; fig. 3.5b) parameters.

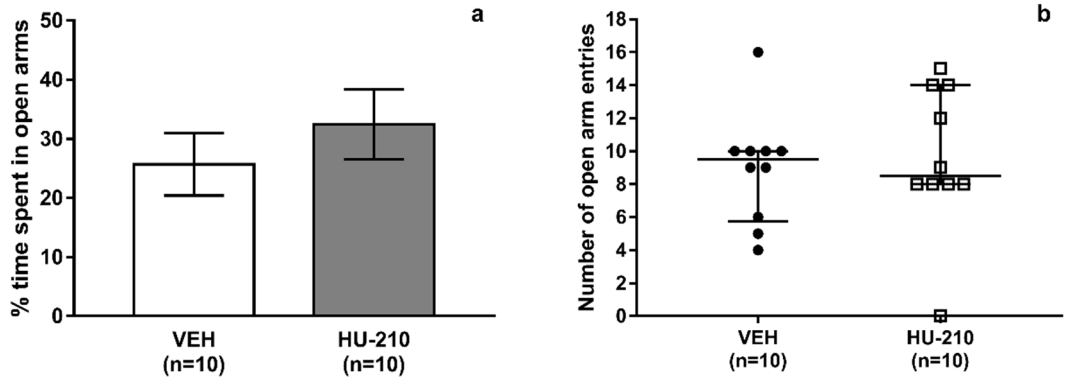

Fig. 3.5 – Chronic adolescent exposure to HU-210 did not persistently alter adult anxiety-like behavior in the EPM after a 27-day drug washout. The EPM was performed on PND 80 with two measures of anxiety being derived from the 5-minute trial, neither of which showed significant differences: percentage of time spent in the open arms (data expressed mean ± SEM [n=10]; unpaired Student's *t*-test) **(a)**; and the total number of open arm entries (data expressed as median and IQR [n=10]; Mann-Whitney U test) **(b)**.

3.2.2 – Open Field Test

3.2.2.1 – Anxiety-like Behavior

To complement the measures obtained with the EPM, the first OFT trial was scored for measures relating to anxiety-like behavior. In this test HU-210-treated rats showed no statistically significant difference from controls, regarding either the percentage of time spent (VEH = 3.18 ± 0.726, HU-210 = 4 ± 0.885; t(18) = 0.716, p = .483), or of distance traveled (VEH = 4.37 ± 0.748, HU-210 = 3.9 ± 0.885; t(18) = 0.551, p = .589) in the CZ of the OF (fig. 3.6).

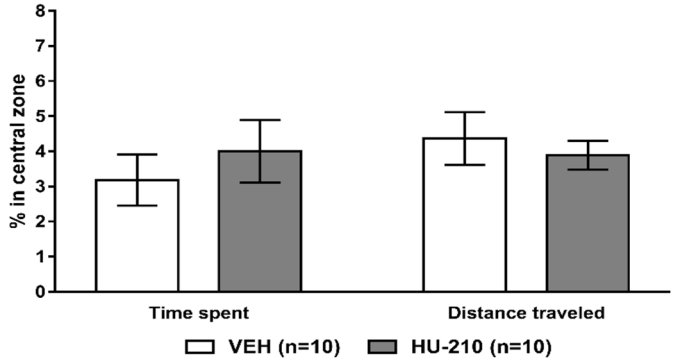

Fig. 3.6 – Chronic adolescent exposure to HU-210 did not persistently alter adult anxiety-like behavior in the OFT after a 27-day drug washout. Two measures of anxiety were derived from the first OFT trial (PND 80): percentage of time spent **(a)**, and distance traveled **(b)** in CZ. Neither parameter showed significant differences across groups. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

3.2.2.2 – Locomotor Activity

To determine if chronic HU-210 exposure during the adolescent period induced persistent alterations in locomotor activity, the second OFT trial was scored for measures relating to this parameter. Specifically, no significant differences between groups were found for either average velocity (VEH = 14.2 ± 1.1, HU-210 = 13.5 ± 0.84; t(17) = 0.542, p = .595; fig. 3.7a), or total distance traveled (VEH = 1588 ± 79.8, HU-210 = 1454 ± 206; t(18) = 0.609, p = .550; fig. 3.7b) during this trial.

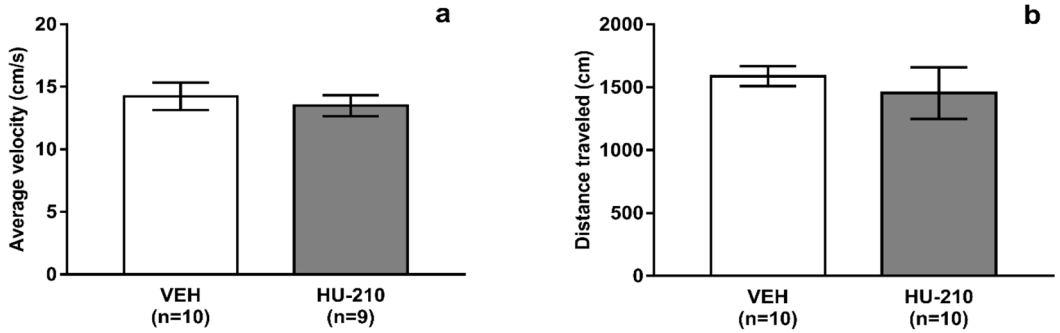

Fig. 3.7 – Chronic adolescent exposure to HU-210 did not persistently alter adult locomotor activity in the OFT after a 27-day drug washout. Two measures of locomotor activity were derived from the second OFT trial (PND 81): average velocity **(a)**, and total distance traveled **(b)** during the entire 10-minute trial. Neither parameter showed significant differences across groups. Data are expressed as mean ± SEM (n=9-10); unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.6).

3.2.3 – Social Interaction Test

Given that previous studies have shown that chronic adolescent exposure to CBRAs induces long-lasting deficits in social interaction, animals were assessed for performance in the SIT. Comparison of the time spent in active social interaction during the test, showed that HU-210-treated rats did not significantly differ from VEH-treated animals, in this parameter (VEH = 91.4 ± 7.93, HU-210 = 108 ± 13.8; t(18) = 1.04, p = .311; fig. 3.8).

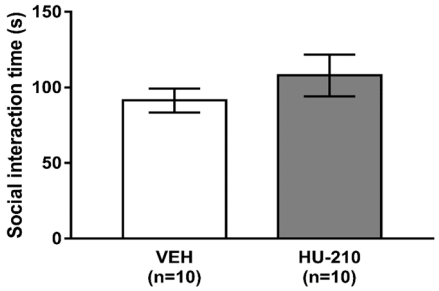

Fig. 3.8 – Chronic adolescent exposure to HU-210 did not persistently alter adult social behavior in the SIT after a 27-day drug washout. Total time spent in active social interaction was quantified during the 10-minute SIT (PND 82). No difference was found between treatment groups Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

3.2.4 – Modified Forced Swim Test

To assess the possibility that chronic HU-210 treatment might lead to persistent alterations at the level of stress-coping behavior, animals were tested in the mFST (fig. 3.9). No differences between groups were found in either the time spent swimming (VEH = 165 ± 6.74, HU-210 = 179 ± 13.1; t(15) = 0.903, p = .381), or in immobility (VEH = 625 ± 9.75, HU-210 = 630 ± 13.4; t(15) = 0.37, p = .763). However, a significant decrease, in the time spent engaging in climbing behavior, was found for HU-210-treated rats, in comparison to controls (VEH = 110 ± 5.05, HU-210 = 90.8 ± 6.31; t(15) = 2.32, p = .035).

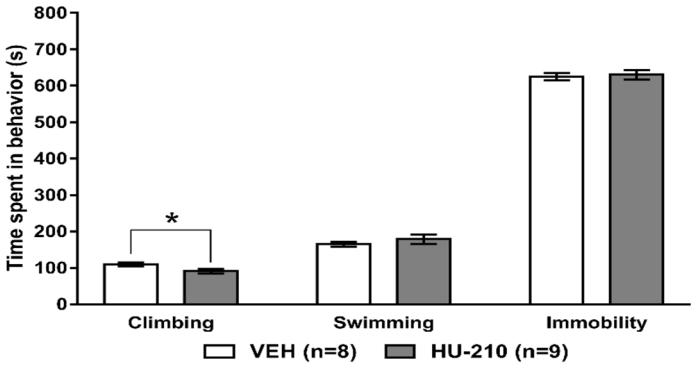

Fig. 3.9 – Chronic adolescent exposure to HU-210 led to persistent alterations of stress-coping behavior in the mFST after a 27-day drug washout. Three parameters were assessed during the 15-minute FST trial (PND 85): climbing, swimming and immobility. While treatment groups did not differ, regarding the time spent swimming or in immobility, HU-210-treated animals spent significantly less time climbing than with controls. Data are expressed as mean ± SEM (n=8-9); * p < .05, unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.6).

3.2.5 – Sucrose Preference Test

To assess the impact of chronic adolescent exposure to HU-210 on adult reward functioning, animals were tested in a 3-day version of the SPT. No differences were found between groups regarding sucrose intake (fig. 3.10a) at either day 1 (VEH = 0.49 ± 0.09, HU-210 = 0.67 ± 0.06; t(18) = 1.69, p = .109), day 2 (VEH = 0.49 ± 0.12, HU-210 = 0.62 ± 0.06; t(18) = 1, p = .329) or day 3 (VEH = 0.38 ± 0.09, HU-210 = 0.51 ± 0.06; t(18) = 1.22, p = .237). Likewise, no differences were found between groups regarding their average daily sucrose intake, over the duration of the experiment (VEH = 0.45 ± 0.09, HU-210 = 0.6 ± 0.05; t(18) = 1.38, p = .183; fig 3.10b).

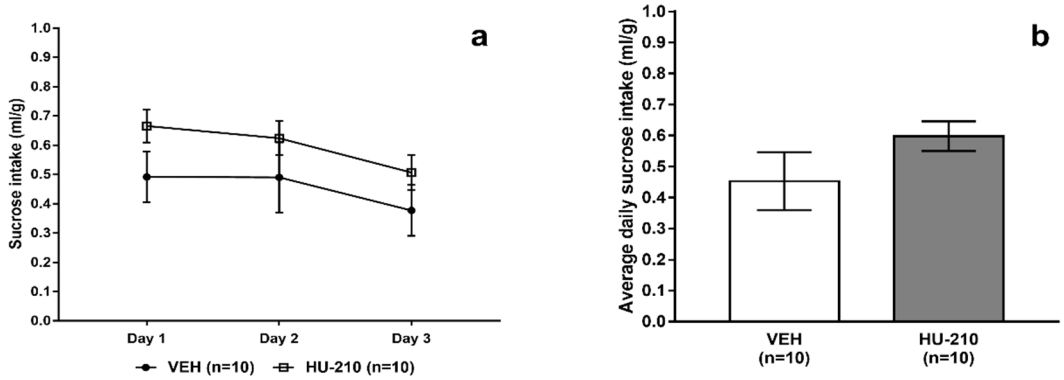

Fig. 3.10 – Chronic adolescent exposure to HU-210 did not persistently alter sucrose intake after a 27-day drug washout. Sucrose intake was assessed daily **(a)** and was averaged at the end of the testing period **(b)**, with no differences being found between groups for either parameter. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

Similarly, comparison of the relative preference for the sucrose solution over water, found no differences between groups at either day 1 (VEH = 94.3 ± 0.98, HU-210 = 95.4 ± 0.36; t(16) = 1.19, p = .251; fig. 3.11a), day 2 (VEH = 94.4, range: 78.2 - 97.2, HU-210 = 95.3, range: 93.6 – 97.2; U = 30, p = .673; fig. 3.11b), day 3 (VEH = 96.3, range: 87.8 – 98.1, HU-210 = 95.7, range: 90 – 98; U = 33.5, p = .589; fig. 3.11c), or over the testing period (VEH = 96.3, range: 89 – 97.6, HU-210 = 95.9, range: 93.5 – 96.7; U = 34, p = .633; fig. 3.11d).

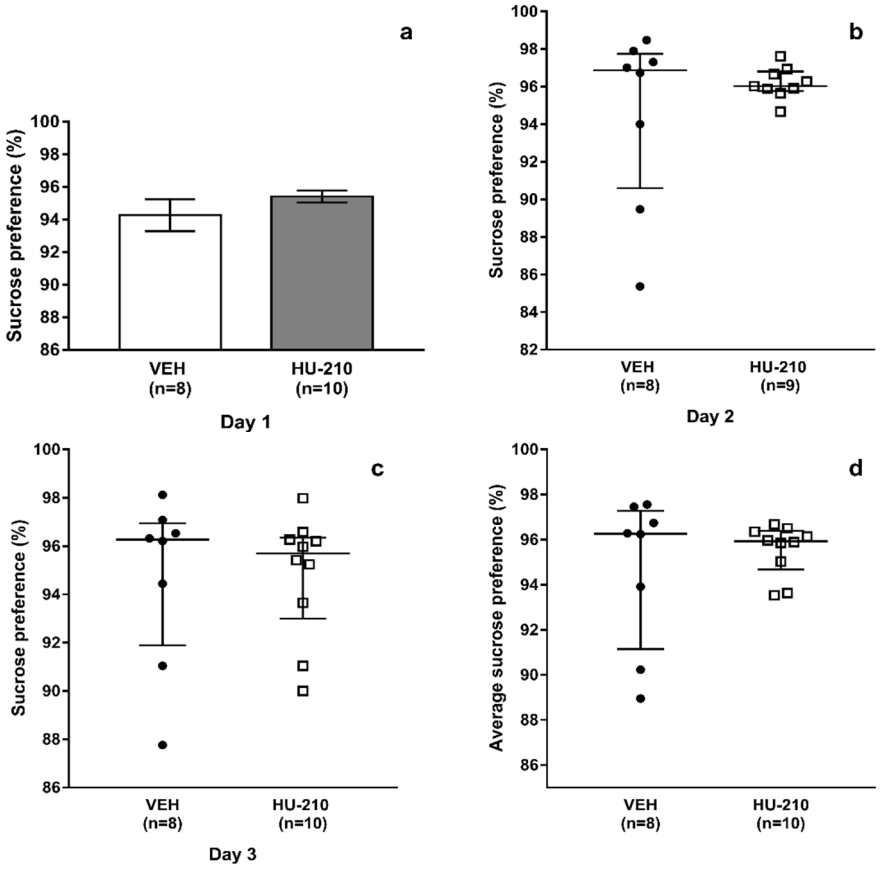

Fig. 3.11 – Chronic adolescent exposure to HU-210 did not persistently alter relative preference for sucrose after a 27-day drug washout. Sucrose preference was assessed daily **(a-c)** and was averaged for the entirety of the testing period **(d)**, with no differences being found between groups for either parameter. Data are expressed as either mean ± SEM (a; n=8-10; unpaired Student's *t*-test) or median and IQR ranges (b-d; n=8-10; Mann-Whitney U test). Reported group sizes exclude significant outliers (see section 2.6).

3.2.6 – Marble Burying Test

Given that the MBT seems, in certain circumstances (see chapter 2), to be a more sensitive assay of anxiety-like behavior, animals were tested in this paradigm, to provide a complementary measure to the EPM and the OFT. Analysis of the number of marbles buried during the 30-minute trial showed no statistically significant difference between groups (VEH = 15.8 ± 1.43, HU-210 = 16.1 ± 1.46; t(18) = 0.147, p = .885; fig. 3.12).

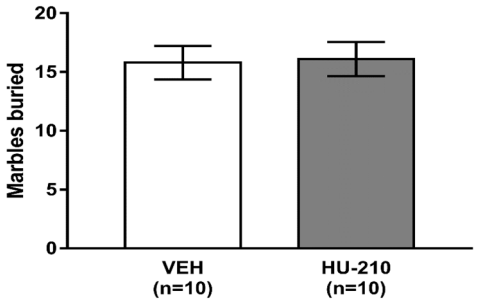

Fig. 3.12 – Chronic adolescent exposure to HU-210 did not persistently alter adult anxiety-like behavior in the MBT after a 27-day drug washout. The number of marbles buried during the 30-minute MBT trial (PND 91) was quantified. No difference was found between groups regarding this measure. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

4 – Discussion

The aim of this experiment was to determine whether chronic adolescent treatment with the highly potent, non-selective, CBRA, HU-210, would lead to persistent alterations in anxiety- and depressive-like behavior at adulthood. While changes in these domains have been repeatedly reported in the literature, as a result of adolescent treatment with other CBRAs, there is a scarcity of data regarding the effects of HU-210. Moreover, the few studies that have studied the effects of prolonged HU-210 exposure, have done so with adult– and not adolescent – animals, and found beneficial effects of treatment[213,390]. Thus, this is the first study explicitly examining the long-term effects of chronic adolescent exposure to this drug, on affective functioning.

Similarly to what has been previously reported for other CBRAs, administration of HU-210 led to a significant decrease in weight gain. This decrease was highly significant during the drug administration period, and remained detectable for 15 days after the end of that period. While this effect is contrary to the widely reported stimulatory effects of CBRAs on food intake in humans[501,502], it has long been reported to exist in rodents[294,300,333,503–505], for reasons not yet entirely clear. Moreover, it should be noted that this effect occurred despite the fact that, at the outset of the experiment, the HU-210 assigned group weighed significantly more than the VEH-treated group – a difference that is entirely attributable to chance, given that animals were randomly assigned to a treatment condition before their first weighing.

Regarding alterations at the level of anxiety-like behavior, HU-210 treatment did not affect behavior in the EPM, with groups showing similar numbers of entries, and percentages of time spent in, the open arms of the maze. Likewise, in the first OFT trial, HU-210-treated animals did not significantly differ from VEH-treated controls in either the time spent, or the distance travelled, in the CZ of the OF. While in accordance with results from other studies with adolescent animals, this lack of anxiogenic effect in the OFT is in disagreement with previous work done with HU-210 in adult animals[493]. This disparity may, however, stem from differences between the two studies regarding the age, strain and sex of the animals, as well as in the dose used. Additionally, in the MBT, the number of marbles buried did not significantly differ as a function of treatment group. Thus, in line with previous animal work with other CBRAs[210,264,265,271,272,290,294–298], these results suggest that chronic adolescent exposure to increasing doses of HU-210 does not alter the expression of anxiety-like behavior at adulthood.

Contrarily to what was expected based on previous reports[261,262,273,274,276,282,308,506], HU-210-treated animals were found to behave similarly to controls in the SIT. That is, whereas chronic adolescent exposure to other CBRAs has been found to lead to decreases in adult social interaction, this type of exposure to HU-210 does not seem to persistently impact social behavior.

Regarding the assessment of depressive-like behavior, when animals were tested in the mFST, the pattern of results obtained differed from those previously reported[210,276,280,282,294,308]. Contrarily to what was expected, based on previous reports, HU-210 treatment did not significantly decrease the time the animals spent either swimming or in immobility. There was, however, a significant decrease in the time animals spent engaging in climbing behavior. Importantly, given that no differences between groups were observed, in either the average velocity, or the total distance traveled in the second OFT trial, it can be argued (but see Stanford et al.[426]) that the differences in the mFST were not the product of alterations at the level of locomotor activity. As such, while there is some suggestion that HU-210 treatment may lead to impaired adult stress-coping, this alteration was not accompanied by increased immobility (the primary parameter by which depressive-like behavior is identified in the mFST) and, thus, cannot be fully interpreted in terms of depressive-like behavior. Moreover, these results are in disagreement with the results reported after adult chronic exposure to HU-210[213,390], thus providing some support to the notion that the timing of exposure to CBRAs is crucial in determining its long-term consequences[496].

Supporting the results obtained with the mFST, HU-210-treated rats tested in the SPT did not significantly differ from controls with regards to either their intake of, or their relative preference for, the sucrose solution over water, both at each time point and over the entirety of the test. Thus, these results suggest that chronic adolescent exposure to HU-210 does not induce persistent deleterious effects on adult reward functioning, contrary to what is the case with other CBRAs[210,276,294,296].

As such, the combined results of the mFST and the SPT can be taken as suggesting that, unlike that previously reported with other CBRAs, chronic exposure to HU-210 during the adolescent period, does not increase adult depressive-like behavior.

There are several, non-mutually exclusive, possible explanations for the discrepancy between the results obtained here, and those previously reported in the literature. The first possibility relates to differences regarding both animal strain and experimental protocol. Whereas most studies on the adult effects of

chronic adolescent exposure to CBRAs – and indeed all studies using the mFST for this purpose – have used Sprague-Dawley rats[210,268,269,282,282,294,294,315], in the present experiment the subjects were of the Wistar strain. This possible confound is further aggravated by the fact that the experimental protocol used introduced several modifications – such as the duration of each dose step and the 2-day periods between dose steps – to the protocol used by Lee et al.[321], which is itself a modified version of the original (and most widely used) protocol, designed by Rubino et al[294]. In addition, the time course of the present experiment was somewhat different, regarding the onset of drug administration, the length of drug washout, and the onset of behavioral testing.

Secondly, there is a possibility that the lack of alterations might not itself be a result of HU-210 ineffectiveness in triggering them, but that drug effects may have been masked by an inadvertent deleterious effect of the vehicle solution. Due to the concentration at which the stock solution was suspended (1mM), an inordinate amount of DMSO was injected in both control and HU-210 animals, to reach appropriate drug dosages. Indeed, DMSO represented 6, 13 and 26% of the total volume injected daily, during the first, second and third drug steps, respectively. Given that prolonged exposure to DMSO has been demonstrated to both be toxic at similar concentrations[507–509], and to induce behavioral alterations[510], it might be the case that the performance of VEH-treated rats was negatively impacted by this exposure. As such, it may be possible that HU-210 treatment *did* negatively impact the behavior of animals, but that this effect was masked by the equally deleterious effects of toxic levels of DMSO. If this were to be the case, it would imply that the few differences found between groups (e.g., the decreased climbing in the mFST) likely result from the additive effect of HU-210 over that of DMSO.

The third possible explanation for these differences relates to the possibility that, notwithstanding the problems previously described, HU-210 might be qualitatively different from the previously studied CBRAs. Indeed, HU-210 differs from THC by being a full, rather than partial, agonist, and from CP 55,940 and WIN 55,212-2 by having different relative affinities for CB_1R and CB_2R. Furthermore, it is also possible that these disparate effects may not be mediated by activity at either CBR, and instead result from non-specific activity at other receptors.

As such, and since multiple possible explanations exist for the unexpected outcomes observed, a second experiment was designed to control for as many of these confounds as possible.

Chapter 4 – Experiment 2

1 – Rationale

Given the numerous confounds that may have led to the unexpected results obtained in experiment 1, the present experiment was designed in such a way as to minimize as many of those confounds as possible. Thus, changes were made regarding the strain of animal used, the number of injections administered to the animals, the duration of the drug administration period, and of each dose step, so as to replicate the protocol that is most commonly used to study the effects of chronic adolescent CBRA exposure [294]. In addition, to diminish the possibility that the vehicle solution may have had a deleterious effect on behavioral performance, a new stock solution of HU-210 was made, at a more appropriate concentration (see section 2.3 below), thus reducing the amount of DMSO administered per injection.

As such, if results show a prodepressant-like effect as a result of HU-210 treatment in these conditions, these would both be in line with previous reports[210,261,262,273,274,276,278,280,282,294,296,306,308,315,506] and, given that similar exposure has been found to be antidepressant in adult animals[213,390], would further support the notion that the timing of exposure is critical with regards to the consequences of chronic cannabinoid use[496]. Conversely, if results are still found to be discrepant with those previously reported for other CBRAs, it would make it more likely that these discrepancies do, in fact, stem from differences in the pharmacology of HU-210, and not from experimental confounds. Moreover, should an antidepressant-like effect be found, this would – in addition to the previous point – also imply that this differential effect of HU-210 *vis a vis* other CBRAs, is age-independent, having also been found in adult animals.

2 – Methods

2.1 – Animals and Ethical Approval

Twenty-five female Sprague-Dawley rats, aged 21 days (PND 21) at the time of arrival, were ordered from Charles River Laboratories (Calco, Italy) and were housed in groups of five, in the same conditions as described in experiment 1. A period of at least three days of acclimatization was allowed before any experimental procedure was performed, and animals were monitored daily for physical and behavioral signs of distress and/or suffering.

All experiments took place during the light phase of the cycle, and were performed in conformity with European Community Guidelines (Directive 2010/63/UE), and with the approval of the Committee for Ethics in Animal Research of the Faculty of Medicine of the University of Lisbon, as well as of the Portuguese Competent Authority for Animal Welfare.

2.2 – Drugs

HU-210 (Tocris Bioscience, Bristol, UK) was suspended in DMSO (Sigma-Aldrich, St. Louis, MO, USA) resulting in a stock solution at 25mM concentration. Aliquots were prepared and stored at -20ºC until the day of use. From this stock solution further dilutions were made each day in 0.9% saline, to reach adequate volume.

2.3 – Drug Administration

At the time of arrival animals were randomly assigned to be treated with either HU-210 (n=10) or vehicle solution (VEH; n=10), or to serve as stimulus animals during the SIT (n=5).

Drug administration (fig. 4.1) was performed according the protocol described by Rubino et al.[294]: Intraperitoneal (i.p.) injections of HU-210 were administered twice-daily for 11 consecutive days, in an escalating dosing schedule (PND 35-37: 25µg/kg; PND 38-41: 50µg/kg; PND 42-45: 100µg/kg), at a volume of 1 ml/kg. At no point during the administration period did DMSO amount exceed 1% of the total injected volume. Injections were administered early in the morning and in the afternoon, with at least 6 hours between them. To minimize animal stress and facilitate injection procedures, animals were handled for at least three days before the first injection took place.

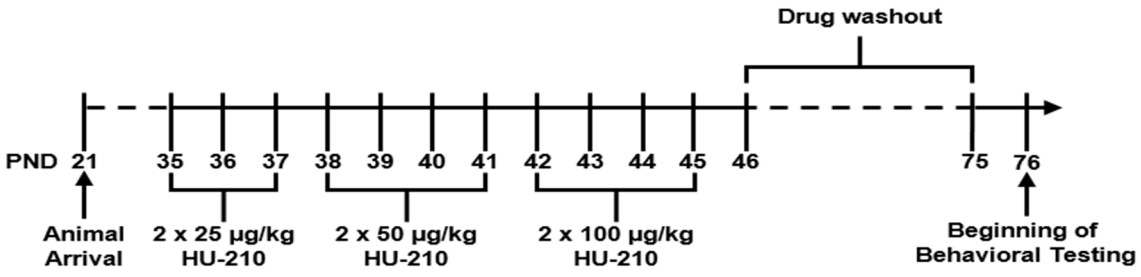

Fig. 4.1 – Timeline of drug administration.

2.4 – Animal Body Weight

Animal body weight was monitored during the entirety of the experiment. During the drug administration period (PND 35-45) animals were weighed daily. To avoid excessively disturbing them, animals were weighed weekly, for the duration of the washout period (PND 46-75). Finally, during the testing period (PND 76-87) animals were weighed at the start of each testing day.

To calculate weight changes, the starting body weight of each animal at PND 28 was subtracted to its body weight at each time-point, with the resulting values (expressed in grams) being used to compare the effects of treatment across groups, at the various stages of the experiment.

2.5 – Behavioral Testing

After drug administration period ended (PND 45), animals were given a 30-day drug washout period, during which they were only disturbed for weekly weighings and periodic cage cleanings. To reduce animal stress, and its possible confounding effect on behavioral performance, rats were individually handled for at least five minutes, on the five days preceding the first test (PND 71-75). Furthermore, to diminish the likelihood of stress induced by one test influencing the results of the following, two-day rest periods were introduced wherever possible. A chronogram of behavioral testing procedures is described in fig. 4.2.

Behavioral testing and analysis procedures were in every way similar to those described in experiment 1.

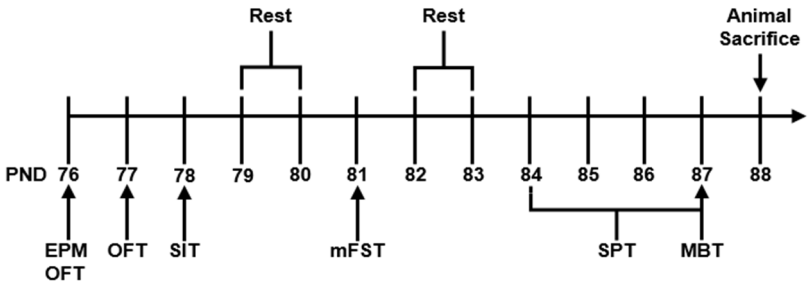

Fig. 4.2 – Chronogram of behavioral experiments performed. PND, post-natal day; EPM, elevated plus maze; OFT, open field test; SIT, social interaction test; mFST, forced swim test; SPT, sucrose preference test; MBT, marble burying test;

2.5.1 – Elevated Plus Maze

The EPM was performed and scored similarly to that described in experiment 1, with the sole difference being that testing occurred on PND 76.

2.5.2 – Open Field Test

The OFT was performed and scored similarly to that described in experiment 1, with the sole difference being that the first trial (scored for anxiety-related parameters) took place on PND 76, and the second trial (scored for locomotor activity related parameters) occurred on PND 77.

2.5.3 – Social Interaction Test

The SIT was performed and scored similarly to that described in experiment 1, with the sole difference being that habituation trials (i.e., the OFT) took place on PND 76-77, and the test trial occurred on PND 78.

2.5.4 – Modified Forced Swim Test

The mFST was performed and scored similarly to that described in experiment 1, with the sole difference being that testing occurred on PND 81.

2.5.5 – Sucrose Preference Test

The SPT was performed and scored similarly to that described in experiment 1, with the sole difference being that the testing occurred from PNDs 84 to PND 87.

2.5.6 – Marble Burying Test

The MBT was performed and scored similarly to that described in experiment 1, with the sole difference being that the testing occurred on PND 87.

2.6 – Western Blot

2.6.1 – Animal Sacrifice and Tissue Removal

On the day following the last behavioral test (PND 88), animals were individually anesthetized with isoflurane, until the paw-pinch reflex was no longer present, and then decapitated.

Brains were quickly removed and dissected in ice-cold artificial cerebral-spinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26mM $NaHCO_3$, 1mM $MgSO_4$, 2mM $CaCL_2$, and 10 mM D-glucose, pH 7.4) previously oxygenated with 95% O_2 and 5% CO_2. Three regions of interest were isolated

in each animal: the hippocampus, striatum and PFC. After isolation, tissue samples were immediately frozen in liquid nitrogen and stored at -80 ºC, until the moment of tissue processing.

2.6.2 – Sample Preparation and Protein Quantification

Whole tissue homogenates were prepared from the samples. For this, samples were homogenized through sonication, in Radio Immunoprecipitation Assay (RIPA) lysis buffer (1% Nonidet® P40 Substitute [NP40], 5 mM ethylenediamine tetraacetic acid [EDTA], 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5), containing one cOmplete™ Mini protease inhibitor cocktail tablet (Roche, Penzberg, Germany) for each 10 ml, and the phosphatase inhibitors 10mM NaF and 5mM Na_3VO_4. Samples were then centrifuged at 16000g for 10 minutes at 4ºC, after which the supernatant was collected.

Sample protein quantification was performed through the Lowry assay, using the DC™ Protein Assay kit (Bio-Rad Laboratories, CA, USA), with bovine serum albumin (BSA) as the standard. Absorbance was read at 750 nm, using an Infinite M200 multimode microplate reader (Tecan, Männedorf, Switzerland). After quantification, samples were prepared for Western Blot, by adding 6x sample buffer (36% glycerol, 12% SDS, 0.015% bromophenol blue, 720 mM dithiothreitol, 420 mM Tris pH 6.8) and denaturing them (10 min, 95ºC), and were then stored at -20ºC until the day of use.

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), in running buffer (0.1% SDS, 192 mM glycine, 25 mM Tris pH 8.3), at constant voltage (80-120 V), using 12% acrylamide/bis-acrylamide resolving gels (0.1% SDS, 0.1% ammonium persulfate [APS], 0.04% N,N,N',N'-tetramethylethane-1,2-diamine [TEMED], 375 mM Tris pH 8.8), and 5% acrylamide/bis-acrylamide stacking gels (0.1% SDS, 0.1% APS, 0.1% TEMED, 125 mM Tris pH 6.8), with 1.5 mm thickness. NZYColour Protein Marker II (NZYTech, Lisbon, Portugal) was used as a protein molecular weight marker. Proteins were transferred to polyvinylidene difluoride (PVDF; GE Healthcare, Buckinghamshire, UK) membranes, previously soaked in methanol, in transfer buffer (10% methanol, 192 mM glycine, 25 mM Tris pH 8.3), at constant amperage (350 mA, 90 min). After transfer, membranes were first soaked in Ponceau S solution (0.1% Ponceau S, 5% acetic acid) to confirm transference efficacy, and were then blocked with 3% BSA in Tris buffered saline with Tween® 20 (TBS-T; 200 nM Tris base, 1.5 M NaCl, 0.1% Tween® 20, pH 7.6) for 1 hour at room temperature (RT). Membranes were washed

with TBS-T (3 x 5 min), before incubation with primary antibodies (overnight at 4ºC), and again before incubation with secondary antibodies (1 hour at RT). All primary (table 4.1) and secondary antibodies (table 4.2) were prepared in the blocking solution. Between incubations with different primary antibodies, membranes were placed in a stripping solution (200 mM glycine, 0.1% SDS, 1% Tween® 20, 50% acetic acid glacial, pH 2.2) for 25 minutes at RT, and washed with TBS-T (3 x 5 min) to remove the previous antibodies.

Table 4.1 – Primary antibodies used for Western Blot.

Antibody	Dilution	Supplier	Reference (RRID)
Guinea pig polyclonal anti-CB_1R	1:500	Frontier Institute Co. Ltd (Hokkaido, Japan)	CB1-GP-Af530 (AB_2571593)
Mouse monoclonal anti-GAPDH	1:5000	Ambion® (CA, USA)	AM4300 (AB_437392)

After incubation with secondary antibodies, membranes were revealed with Western Lightning ECL Pro™ (PerkinElmer, MA, USA), and immunorreactivity was visualized using a Chemidoc XRS+ system (Bio-Rad Laboratories, CA, USA) with Image Lab™ software (Bio-Rad Laboratories, CA, USA). Band intensities were quantified via digital densitometry, through ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band intensities as a loading control. Data were normalized for the VEH-treated group, and are expressed as protein levels (% of VEH).

Table 4.2 – Secondary antibodies used for Western Blot.

Antibody	Dilution	Supplier	Reference (RRID)
Goat polyclonal anti-guinea pig IgG-HRP	1:10000	Santa Cruz Biotechnology (TX, USA)	sc-2438 (AB_650492)
Goat polyclonal anti-mouse IgG-HRP	1:10000	Bio-Rad Laboratories (CA, USA)	172-1011 (AB_11125936)

2.7 – Statistical Analysis

Statistical analysis was in every way similar to that described in experiment 1. All data are expressed as means ± SEM, or as medians and IQR, or range (minimum and maximum values, wherever appropriate), depending on whether parametric or non-parametric tests were used, respectively. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

3 – Results

3.1 – Animal Body Weight

Baseline animal body weight, measured on PND 28, was found to not be significantly different between treatment conditions (VEH = 89.7 ± 3.25, HU-210 = 96.1 ± 2.85; $t(18) = 1.48$, $p = .156$). Moreover, change in weight, relative to PND 28, on the first day of injections (PND 35) was also found to not differ between groups. However, after the first day of injections, and for the duration of the drug administration period (PND 36-45), a highly significant ($p \leq .01$, PNDs 36, 41 and 42; $p \leq .001$ PNDs 37-40 and 43-45) difference in weight change was found between groups, whereby HU-210-treated animals gained significantly less weight than the VEH-treated group (fig. 4.3). This effect persisted for the next 15 days, being detectable at PND 53 ($p \leq .001$) and PND 60 ($p \leq .01$), after which it ceased to be significant, remaining so for the rest of the experiment.

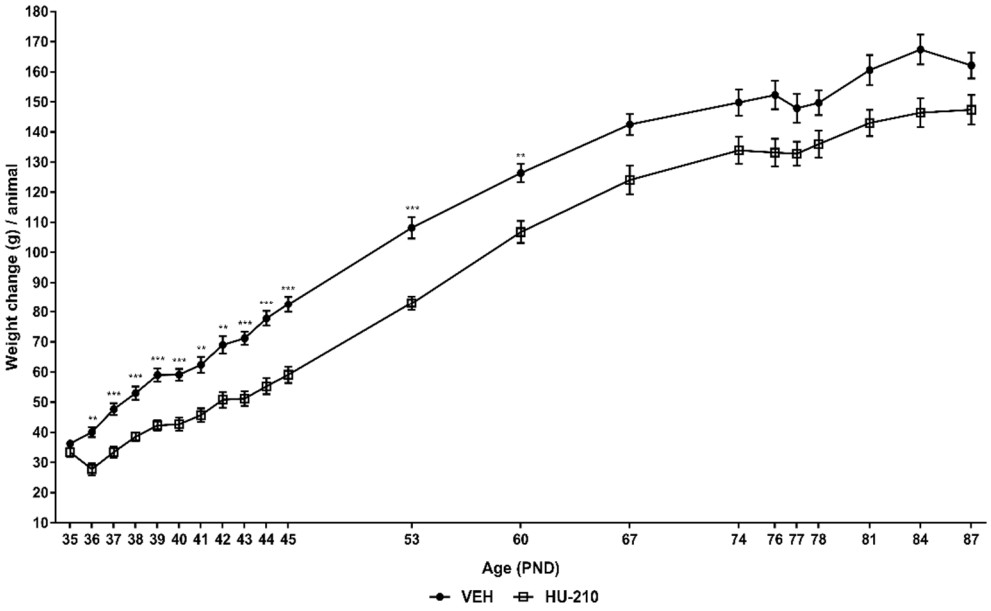

Fig. 4.3 – Change in animal weight relative to PND 28 over the course of the experiment. HU-210 treatment significantly reduced the amount of weight animals gained during the drug administration period (PNDs 36-45), and for 15 days after its end (PND 60). This effect did not, however, persist for the remainder of the experiment. Data are expressed as mean ± SEM (n=10); ** $p \leq .01$, *** $p \leq .001$, unpaired Student's *t*-test with Holm-Sidak correction.

3.2 – Behavioral Testing

3.2.1 – Elevated Plus Maze

To determine whether chronic adolescent HU-210 exposure – following the protocol described by Rubino et al.[294] – impacted adult anxiety-like behavior, both the percentage of time spent, and the number of entries, in the open arms of the EPM were compared across groups. As was the case in the first

experiment, the HU-210 group did not significantly differ from VEH-treated controls in either the first (VEH = 42.5 ± 5.06, HU-210 = 39.1 ± 5.26; t(17) = 0.463, p = .650; fig. 4.4a) or second parameters (VEH = 10.8 ± 0.48, HU-210 = 9.13 ± 0.69; t(12) = 1.89, p = .084; fig. 4.4b).

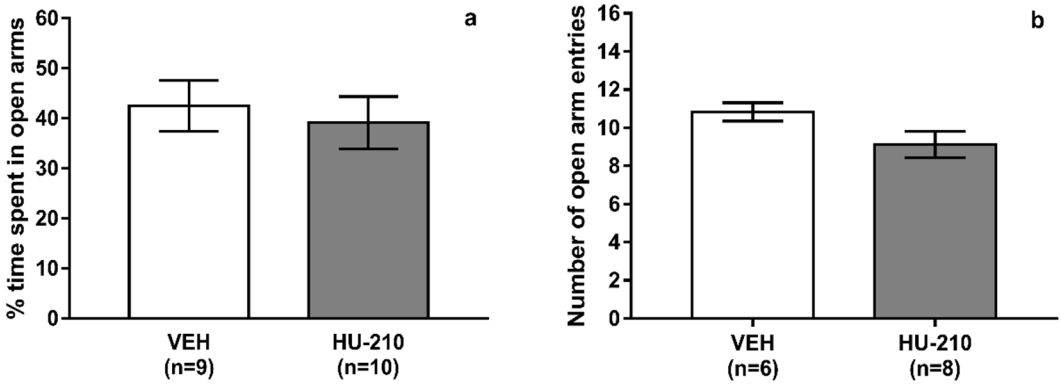

Fig. 4.4 – Chronic adolescent exposure to HU-210 did not persistently alter adult anxiety-like behavior in the EPM after a 30-day drug washout. The EPM was performed on PND 76 with two measures of anxiety being derived from the 5-minute trial, neither of which showed significant differences: percentage of time spent in the open arms **(a)**, and the total number of open arm entries **(b)**. Data are expressed as means ± SEM (n=6-10); unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.7).

3.2.2 – Open Field Test

3.2.2.1 – Anxiety-like Behavior

Similarly to what was done on the first experiment, the first OFT trial was assessed for parameters relating to anxiety-like behavior. Specifically, as had been found in experiment 1, no differences between groups were found, regarding either the percentage of time spent (VEH = 7.58 ± 1.32, HU-210 = 6.58 ± 0.851; t(18) = 0.642, p = .529), or distance traveled (VEH = 8.31 ± 1.08, HU-210 = 8.93 ± 0.92; t(18) = 0.436, p = .668) in the OFT CZ (fig. 4.5).

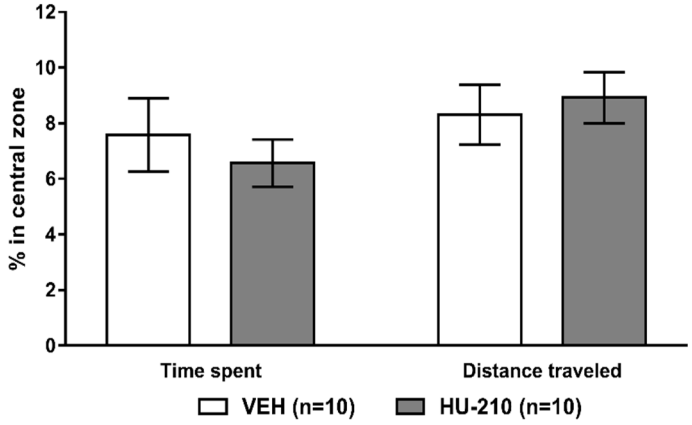

Fig. 4.5 – Chronic adolescent exposure to HU-210 did not persistently alter adult anxiety-like behavior in the OFT after a 30-day drug washout. Two measures of anxiety were derived from the first OFT trial (PND 76): percentage of time spent **(a)**, and distance traveled **(b)** in CZ. Neither parameter showed significant differences across groups. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

3.2.2.2 – Locomotor Activity

To determine if the differences between the experimental protocols used in the first and second experiments reflected themselves on the locomotor activity of the animals, the second OFT trial was scored for measures relating to this parameter. Specifically, no changes were found in either average velocity (VEH = 14.9 ± 0.81, HU-210 = 14 ± 0.75; t(18) = 0.864, p = .399; fig. 4.6a) or total distance traveled (VEH = 1752 ± 77.2, HU-210 = 1748 ± 118; t(18) = 0.027, p = .979; fig. 4.6b), over the 10-minute trial, as a function of treatment.

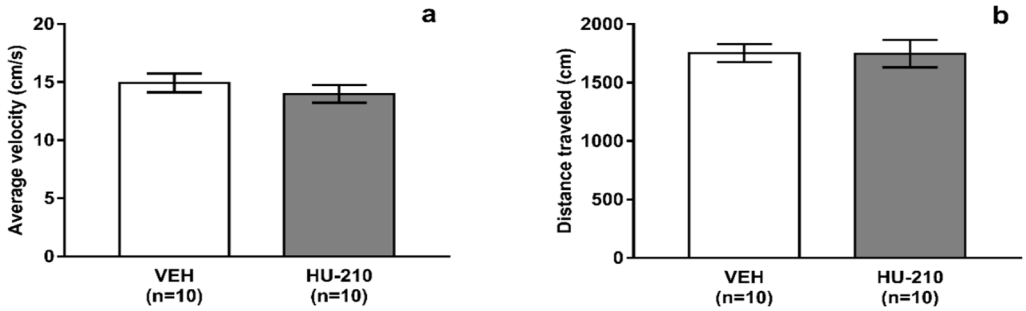

Fig. 4.6 – Chronic adolescent exposure to HU-210 did not persistently alter adult locomotor activity in the OFT after a 30-day drug washout. Two measures of locomotor activity were derived from the second OFT trial (PND 77): average velocity **(a)**, and total distance traveled **(b)** during the duration of the 10-minute trial. Neither parameter showed significant differences across groups. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

3.2.3 – Social Interaction Test

Since previous studies using the protocol described by Rubino et al.[294], have found that chronic adolescent exposure to CBRAs induces deficits in SIT performance[261,262,273,274,276,282,308,506], and these deficits were not found in experiment 1, animals in this experiment were also tested in this assay.

Contrary to previous studies, and in accordance with the outcomes observed in experiment 1, HU-210-treated animals behaved similarly to controls, with no significant differences in social interaction time being found (VEH = 106 ± 7.43, HU-210 = 122 ± 9.62; t(18) = 1.28, p = .217; fig. 4.7).

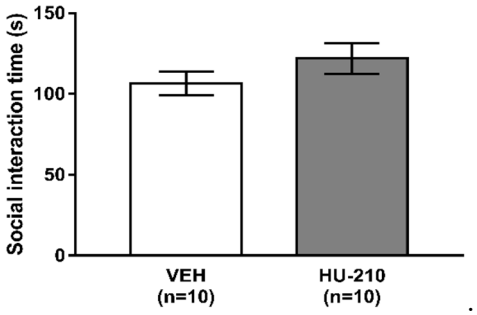

Fig. 4.7 – Chronic adolescent exposure to HU-210 did not persistently alter adult social behavior in the SIT after a 30-day drug washout. Total time spent in active social interaction was quantified during the 10-minute SIT (PND 78). No difference was found between treatment groups. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

3.2.4 – Modified Forced Swim Test

In the first experiment some alterations were found that suggested impairments at the level of stress-coping/depressive-like behavior. However, the classical sign of depressive-like behavior in the mFST – increases in the time spent in immobility[448] – was not observed. Thus, in the present experiment the mFST was also performed, with the intent of trying to determine if the mixed results previously obtained derived from the confounds identified in experiment 1.

Surprisingly, no differences were found between treatment groups (fig. 4.8), regarding either the time the animals spent climbing (VEH = 104 ± 9.49, HU-210 = 100 ± 10.9; t(17) = 0.266, p = .793), swimming (VEH = 162 ± 6.67, HU-210 = 142 ± 17.5; t(17) = 1.08, p = .297), or in immobility (VEH = 634 ± 11.8, HU-210 = 658 ± 17; t(17) = 1.14, p = .27).

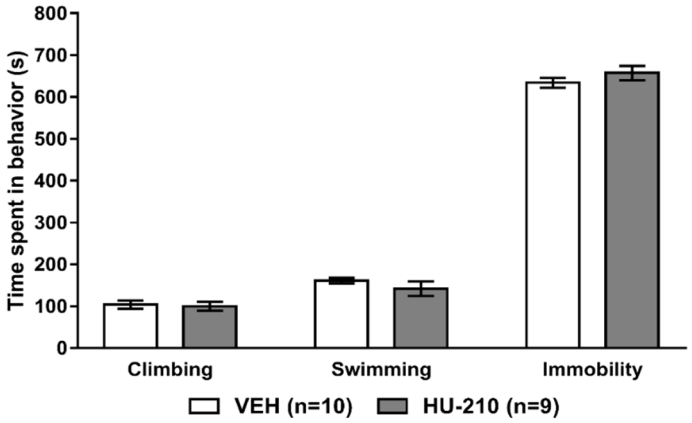

Fig. 4.8 – Chronic adolescent exposure to HU-210 did not persistently alter stress-coping behavior in the mFST after a 30-day drug washout. Three parameters were assessed during the 15-minute FST trial (PND 81): climbing, swimming and immobility. Treatment groups did not differ regarding the time spent any of these behaviors. Data are expressed as mean ± SEM (n=9-10); unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.7).

3.2.5 – Sucrose Preference Test

Because no alterations were found in SPT behavior in the first experiment, despite previous studies of chronic adolescent CBRA exposure having found them[210,276,294,296], the test was again performed in the present experiment.

Similar to the results obtained in experiment 1, comparisons of sucrose intake between groups (fig. 4.9a), found no group differences for days 1 (VEH = 0.36 ± 0.06, HU-210 = 0.32 ± 0.05; t(18) = 0.542, p = .595), 2 (VEH = 0.25 ± 0.04, HU-210 = 0.23 ± 0.05; t(18) = 0.359, p = .724) or 3 (VEH = 0.27 ± 0.05, HU-210 = 0.24 ± 0.05; t(18) = 0.358, p = .725). Furthermore, the average daily sucrose intake over the

duration of the experiment was also not significantly different between groups (VEH = 0.29 ± 0.05, HU-210 = 0.26 ± 0.05; t(18) = 0.447, p = .660; fig. 4.9b).

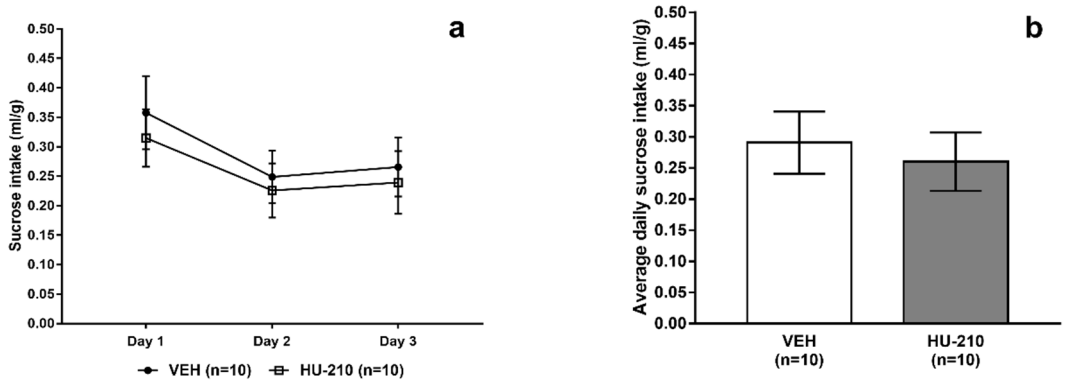

Fig. 4.9 – Chronic adolescent exposure to HU-210 did not persistently alter sucrose intake after a 30-day drug washout. Sucrose intake was assessed daily **(a)** and was averaged at the end of the testing period **(b)**, with no differences being found between groups for either parameter. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

Likewise, comparison of the preference for the sucrose solution over water at each time point (fig. 4.10a), also found no differences between groups for either the first (VEH = 90.6, range: 80 – 95.7, HU-210 = 90.3, range: 73.7 – 95.3; U = 44.5, p = .699), second (VEH = 90.1, range: 74.4 – 92.9, HU-210 = 86.1, range: 23.5 – 97.2; U = 39, p = .661) or third days (VEH = 88.1, range: 67.5 – 92.4, HU-210 = 87.2, range: 78.1 – 93.4; U = 35, p = .942). Additionally, the averaged preference over the duration of the experiment (VEH = 89.9, range: 78.3 – 93.2, HU-210 = 87.3, range: 45.7 – 93.8; U = 36, p = .497; fig. 4.10b) also did not significantly differ across groups.

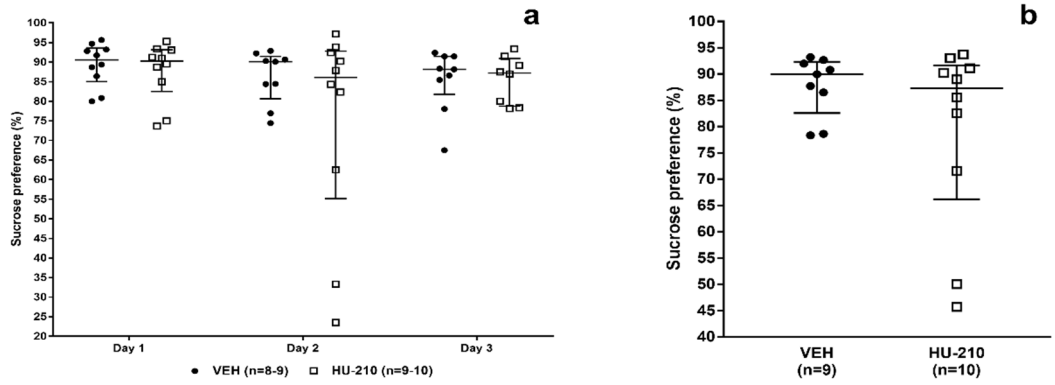

Fig. 4.10 – Chronic adolescent exposure to HU-210 did not persistently alter relative preference for sucrose after a 30-day drug washout. Sucrose preference was assessed daily **(a)** and was averaged for the entirety of the testing period **(b)**, with no differences being found between groups for either parameter. Data are expressed as medians and IQR (n=8-10); Mann-Whitney U test. Reported group sizes exclude significant outliers (see section 2.7).

3.2.6 – Marble Burying Test

As was the case in the first experiment, the MBT was also performed in this group of animals, as a novel measure of the effect of chronic adolescent CBRA exposure, on adult anxiety-like behavior.

Similarly to the results previously obtained, the number of marbles buried did not significantly differ between groups (VEH = 15.4 ± 1.34, HU-210 = 13.5 ± 1.39; t(18) = 0.982, p = .339; fig. 4.11), in line with the behavioral outcomes observed in both the EPM and the first OFT trial.

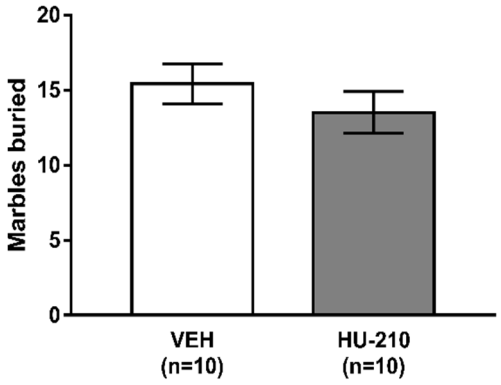

Fig. 4.11 – Chronic adolescent exposure to HU-210 did not persistently alter adult anxiety-like behavior in the MBT after a 30-day drug washout. The number of marbles buried during the 30-minute MBT trial (PND 87) was quantified. No difference was found between groups regarding this measure. Data are expressed as mean ± SEM (n=10); unpaired Student's *t*-test.

3.3 – Western Blot

To assess the possible alterations that chronic adolescent exposure to HU-210 may induce on the expression of CB_1R, the protein levels of these receptors were quantified, in three brain regions known to be involved in affective functioning.

Congruently with the lack of behavioral alterations, no changes in CB_1R protein levels were found in either the hippocampus (VEH = 100 ± 23.7, HU-210 = 84.5 ± 14.8; t(8) = 0.582, p = .579; fig. 4.12a), the striatum (VEH = 100 ± 29.7, HU-210 = 71.7 ± 6.74; t(8) = 0.929, p = .380; fig. 4.12b), or the PFC (VEH = 100 ± 27.8, HU-210 = 110 ± 19.6; t(8) = 0.3, p = .772; fig. 4.12c) of HU-210-treated rats.

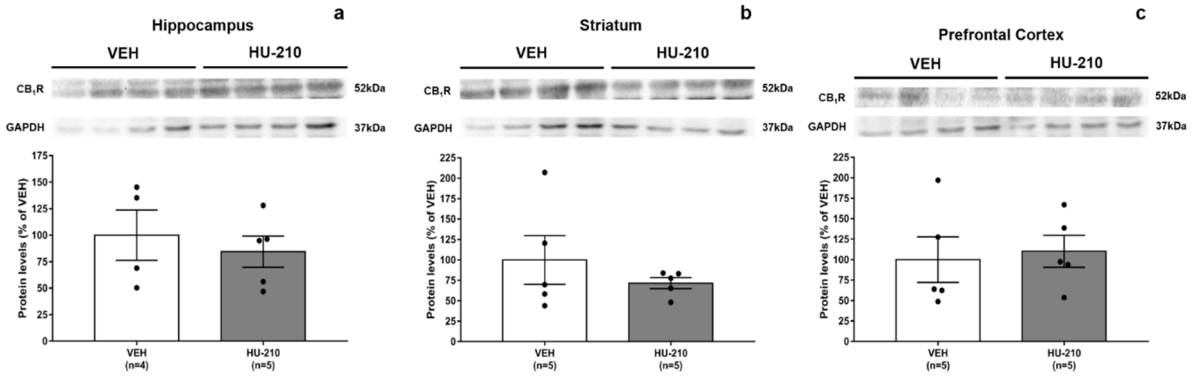

Fig. 4.12 – Chronic adolescent exposure to HU-210 did not persistently alter CB_1R protein levels, in regions involved in affective functioning, after a 30-day drug washout. The levels of CB_1R protein were quantified through Western Blot. No differences were found in either the hippocampus **(a)**, the striatum **(b)** or the PFC **(c)** in the HU-210-treated group. Data are expressed as mean ± SEM (n=4-5); unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.7).

4 – Discussion

Given that the pattern of results obtained in experiment 1 was markedly different from that previously described[210,261,262,273,274,276,278,280,282,294,296,306,308,315,506], and that these differences may have stemmed from experimental confounds – such as the possible deleterious effects of high volumes of DMSO – the present experiment was designed with the intent of minimizing as many of those confounds as possible. Moreover, to further increase the comparability of results, drug administration was performed in accordance with the most widely described experimental protocol in this literature[294]. It is, thus, surprising, that the results obtained in these conditions are not only at even greater odds, with what had been described for other CBRAs[210,261,262,273,274,276,278,280,282,294,296,306,308,315,506], than those obtained in the first experiment, but also with those reported for adult HU-210 exposure[213,390].

As in experiment 1, HU-210 treatment led to a marked decrease of weight-gain during the administration period. Moreover, replicating those results, this effect persisted for 15 days after HU-210 administration ended, but it ceased to be noticeable at the following time-points. The fact that not only did this effect manifest in both experiments, but did so in the exact same timeframes, strongly suggests that it is, indeed, a product of HU-210 activity and not an artifact of experimental confounds.

Regarding anxiety-like behavior, the results obtained are in accordance with both those of the first experiment, and those of the previous literature in adolescent animals. That is, chronic adolescent HU-210 exposure did not alter the performance of the animals in either the EPM, the OFT, or the MBT. Moreover, whereas the first two tests had been previously used in this literature, the MBT had yet not been performed. As such, while the results from this test in the first experiment could have stemmed from the aforementioned experimental confounds, the results we present here – by being free of those methodological concerns – represent a novel finding in this field. Additionally, the fact that, as in experiment 1, but unlike in previous adult work with this drug[493], decreases in the time spent in the CZ of the OF were not found, may be explained either by differences in age, strain and sex of the animals, as well as in the doses used.

In alignment with the results from experiment 1, but in stark contrast with those of the literature, no effects were found at the level of social interaction behavior, as indexed by the SIT, after chronic adolescent exposure to HU-210. Whereas these results could have been attributed to experimental confounds in the

first experiment, in the present case this is not a likely explanation, thus showing a clear difference between HU-210 and other CBRAs.

Similarly, analysis of the mFST data revealed no differences in the performance of HU-210-treated animals. This result differs from that of the first experiment, where a significant decrease in climbing behavior was found. Moreover it is also in contrast with the previous literature where increases in immobility and decreases in swimming behavior are generally detected[276,280,282,308,315]. Thus, it seems to be the case that chronic adolescent exposure to HU-210 does not lead to lasting deficits at the level of stress-coping behavior. Furthermore, these results are also at odds with those reported for chronic adult HU-210 exposure, where antidepressant-like effects were found[213,390]. While this discrepancy may stem from a difference in the drug dosages used – indeed, Jiang et al.[213] exclusively administered the highest dose used in the present experiment (100 μg/kg) – it may also be indicative of the well-established age-dependent differences in long-term CBRA exposure effects[496]. However, there is a possibility that mFST performance may have been contaminated by behavioral instability during the trial. Indeed, despite the fact that most of the previous studies on the affective impact of chronic adolescent exposure to HU-210 only perform one 15-minute mFST session[276,280,282,308,315], it is advised that, when doing this test with rats, two sessions be performed – with only the second session, lasting 5 minutes, being recorded and scored – so as to obtain more stable patterns of behavior[450].

Notwithstanding this caveat, and in accordance with the unaltered behavior in the mFST, SPT performance was also unchanged as a result of treatment. These results, while in line with those of previously obtained in experiment 1, are at odds with those of the literature, where chronic adolescent CBRA exposure has been shown to lead to impairments in reward functioning, as indexed by this test[210,276,294,296]. As such, the combined results of the mFST and SPT, can be interpreted as meaning that – at least when following the most widely used experimental protocol – chronic adolescent exposure to HU-210 does not lead to lasting increases in adult depressive-like behavior, in contrast with adolescent exposure to other CBRAs, such as THC.

Congruently with the lack of behavioral effects, western blotting revealed no differences in the levels of CB_1R protein in either the hippocampus, the striatum or PFC of HU-210-treated animals. While these results are in agreement with two other studies using the same technique[328,330], two caveats must be made:

for one, studies using western blotting have also found chronic adolescent CBRA exposure to lead to increases in hippocampal[329], striatal[328], or prefrontal[282] CB_1R protein levels. Secondly, while the lack of differences observed here is in accordance with the results obtained by most studies using immunohistochemistry/fluorescence techniques[305,312,320,322], it must be noted that a large number of reports employing radioligand binding assays – a much more reliable and precise methodology for the quantification of receptor levels – found chronic adolescent CBRA treatment to lead to significant decreases in CB_1R density[280,282,291,294,333,334]. One possible reason for this difference may be that, while radioligand assays commonly use only the membranar fraction of the tissue homogenate, here this was not done: thus, it may have been the case that chronic adolescent exposure to HU-210 did lead to a decrease in the number of membrane bound receptors through internalization, but that this was not detected, as these internalized receptors would still be detectable in the whole-tissue homogenate.

Overall, the results obtained in this experiment are highly surprising, given both the structural similarity, increased potency, efficacy and affinity for CB_1R, of HU-210 in comparison to THC[373,375,511]. This is even more the case, when taking into account that these finding differ not only from the literature with other drugs, but, indeed, from those obtained in experiment 1. That is, whereas a decrease in climbing behavior was found in experiment 1, no alteration was observed in any mFST parameter in the present experiment. On the one hand, the latter disparities can largely be attributed to differences in the experimental protocol and animal strain used, as well as to the possible deleterious effects of high quantities of DMSO. On the other hand, given that the only detectable difference, between the present study and those previously published, concerns the drug used, the most likely explanation for these discrepant results is that HU-210 may be qualitatively different from other CBRAs. If this were the case, this qualitative difference may stem from differences in affinity, potency and/or efficacy for CBRs[373,375,511], from differences in the intracellular signaling mechanisms triggered by CBR activation[376,391], from pharmacokinetic differences, or from non-specific interactions with other pharmacological targets[293]. Confirmation of these differences would be an important finding for studies employing CBRAs, given that it is fairly commonplace that researchers will look at results obtained with different drugs as being comparable, when it might actually be the case that they present specific characteristics that not only prevent that comparison, but actually lead to the formation of erroneous assumptions about drug effects.

This is even more relevant when considering the high degree of structural similarity between HU-210 and THC: that is, if two highly similar drugs lead to such different results, it is not unreasonable to expect that this would be even more the case for structurally dissimilar drugs, such as WIN 55,212-2 or CP 55,940.

Notwithstanding the specific mechanistic differences of HU-210 *vis a vis* other CBRAs, a question remains as to how those differences manifest themselves, to lead to the lack of alterations observed. Specifically, two options present themselves as the most likely: for one HU-210 may have no long-term effects because whatever changes it is capable of inducing, then revert to normality during the washout period. Alternatively, this lack of effects may indicate that – despite HU-210 having a demonstrable impact on adult stress-coping behavior – adolescent animals are partially or completely resistant to the affective effects of HU-210.

To disentangle these two possibilities, and given that adult studies have shown HU-210 treatment to also be antidepressant immediately after the end of the administration period, a third experiment was designed, where the mFST was performed on the day following the end of the drug administration period. Furthermore, to control for the possible confounding effect of behavioral instability in the first mFST session, the full two-session protocol was used.

Chapter 5 – Experiment 3

1 – Rationale

In the previous experiment, chronic adolescent exposure to increasing doses of HU-210, was found to have no lasting behavioral effects, in contrast with what has been described for both adolescent exposure to other CBRAs[210,261,262,273,274,276,278,280,282,294,296,306,308,506], and adult exposure to HU-210[213,390]. That is, the results of experiment 2 suggest that not only is HU-210 different from other CBRAs in its long-term effects, but also that – as is the case for these other drugs[496] – these effects are age-dependent. Both of these aspects present interesting lines of inquiry, and the present experiment was designed in such a way as to allow their simultaneous exploration.

To this end, a new batch of animals was treated with the exact same administration protocol used in experiment 2, but – instead of allowing them to washout from the drugs – was tested for alterations in the stress-coping dimension of depressive-like behavior (i.e., in the mFST), on the two days immediately following the last drug exposure.

On the one hand, by replicating, in adolescent animals, previous work done with adults exposed to HU-210[390], the results of this experiment allow for further exploration of the role that age of exposure plays in the long-term consequences of drug treatment. On the other hand, by testing adolescent animals immediately after drug exposure ceased, this experiment helps to elucidate how the putative pharmacological differences, between HU-210 and other CBRAs may lead to the lack of adult effects observed in experiment 2. Furthermore, by using the full 2-session mFST protocol, the present experiment additionally allows for the determination of the role that behavioral instability during the first mFST session[450] may have played, in the outcomes observed in this test, in experiments 1 and 2.

As such, several different sets of hypothesis exist for this experiment. First, when considering the apparent differences between HU-210 and other CBRAs, finding altered mFST behavior in the present experiment would support the notion that HU-210 is capable of inducing behavioral alterations, but that these cease to be detectable after a period of abstinence. Conversely, finding no such effects, even in the immediate aftermath of drug administration, would support the notion that – in contrast to what is the case

for other CBRAs – adolescent animals are less vulnerable to HU-210-induced behavioral alterations[260,496,512,513].

Secondly, when considering the role that age may play in the effects of prolonged HU-210 exposure, if animals are found to behave similarly to that described by Morrish et al.[390] in adult rats (i.e., antidepressant-like behavior), this would suggest that – at least in the immediate – HU-210 affects behavior in an age-independent manner. By contrast, finding a different outcome, such as no behavioral alteration or an increase in depressive-like behavior, would suggest that prolonged exposure to HU-210 leads to different outcomes, depending on the age at which takes place, as is the case for other CBRAs[496].

Finally, if behavioral instability in the first mFST session is capable of biasing results, then it would be expected that, here, performance in the first and second test sessions would be noticeably different. On the contrary, if performance is found to be similar, this would imply that behavioral instability likely had a limited (if at all relevant) effect on mFST outcomes reported not just in experiments 1 and 2, but also in previous studies.

2 – Methods

2.1 – Animals and Ethical Approval

Twenty female Sprague-Dawley rats, aged 21 days (PND 21) at the time of arrival, were ordered from Charles River Laboratories (Calco, Italy) and were housed in groups of five, in the same conditions as described in experiment 2. A period of at least three days of acclimatization was allowed before any experimental procedure was performed, and animals were monitored daily for physical and behavioral signs of distress and/or suffering.

All experiments took place during the light phase of the cycle, and were performed in conformity with European Community Guidelines (Directive 2010/63/UE), and with the approval of the Committee for Ethics in Animal Research of the Faculty of Medicine of the University of Lisbon, as well as of the Portuguese Competent Authority for Animal Welfare.

2.2 – Drugs

The same HU-210 stock solution at 25mM concentration used in experiment 2 was used here. From this stock solution further dilutions were made each day, in 0.9% saline, to reach adequate volume.

2.3 – Drug Administration

At the time of arrival animals were randomly assigned to be treated with either HU-210 (n=10) or vehicle solution (VEH; n=10).

Drug administration (fig. 5.1) was performed in every way similarly to that described for experiment 2.

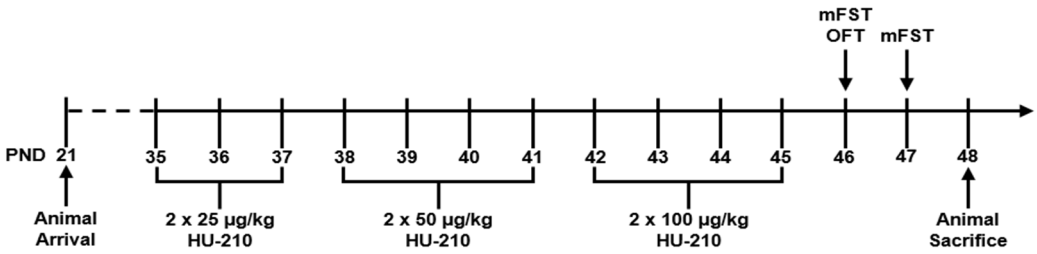

Fig. 5.1 – Chronogram of drug administration and behavioral testing. mFST, modified forced swim; OFT, open field test.

2.4 – Animal Body Weight

Animal body weight was monitored during the entirety of the experiment, with animals being weighed daily for the full period of drug administration and behavioral testing (PND 35-47).

To calculate weight changes, the starting weight of each animal (PND 28) was subtracted to its weight at each time-point, with the resulting values (expressed in grams) being used to compare the effects of treatment on weight-gain across groups.

2.5 – Behavioral Testing

The day after drug administration period ended (PND 46), animals were subjected to behavioral testing, so as to assess the immediate effects of chronic adolescent HU-210 exposure. Because any effect that might exist was judged likely to disappear over time, only two tests were used: the OFT, as a measure of locomotor activity and anxiety-like behavior, and the mFST, with the full two session protocol (fig. 5.1). The mFST was chosen because it is the most widely used animal behavioral test of depressive-like behavior, whereas the OFT was chosen to control for possible locomotor alterations that could bias the results of the former test.

To reduce animal stress and its possible confounding effect on behavioral performance, rats were individually handled for at least five minutes, on the five days preceding the first test (PND 41-45), after morning injections.

Behavioral testing and analysis procedures were in every way similar to those described in experiment 1.

2.5.1 - Open Field Test

Unlike in the previous experiments, only a single 10-minute OFT trial was performed, taking place on PND 46. This trial was scored for parameters relating to both anxiety and locomotor activity. Specifically, for the assessment of anxiety, permanence time and distance traveled in CZ (expressed as percentages of total time, and distance travelled, respectively) were taken as inverse indexes of anxiety. For the assessment of locomotor activity, average velocity (expressed as cm/s) and total distance traveled (expressed as cm), were taken as indexes of this parameter.

2.5.2 – Modified Forced Swim Test

The mFST was performed as described for experiments 1 and 2, with the difference that a second 5-minute session was also performed, 24 hours after the first 15-minute trial. Both sessions were recorded and scored for the parameters previously described. The first session was performed on PND 46, immediately after the end of the OFT, and the second session was performed on PND 47.

2.6 – Western Blot

2.6.1 – Animal Sacrifice and Tissue Removal

On the day following the last behavioral test (PND 48), animals were individually anesthetized with isoflurane, until the paw-pinch reflex was no longer detectable and were then decapitated.

The process of brain removal and dissection, as well as the regions isolated, and the way samples were stored, were in every way similar to that described in experiment 2.

2.6.2 – Sample Preparation and Protein Quantification

Sample preparation, protein quantification and western blotting procedures were in every way similar to those described in experiment 2.

2.7 – Statistical Analysis

Statistical analysis was in every way similar to that described in experiments 1 and 2. Additionally, to assess the degree of correlation between behavior in the first and second mFST sessions, Pearson correlation (r) coefficients were obtained.

All data are expressed as means ± SEM, or as medians and IQR, or range (minimum and maximum values, wherever appropriate), depending on whether parametric or non-parametric tests were used, respectively. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

3 – Results

3.1 – Animal Body Weight

Animal body weight was measured at PND 28, at which point no difference was found between animals assigned to be treated with vehicle solution and those assigned to HU-210 treatment (VEH = 124 ± 1.42, HU-210 = 125 ± 1.26; $t(18) = 0.528$, $p = .604$). On the first day of the drug administration period (PND 35) there was no significant difference between treatment groups, regarding weight-gain relative to PND 28. However, 24 hours after the first injection (PND 36), and for the remainder of the experiment, the HU-210-treated animals showed highly significant decreases (all $p \leq .01$) in weight gain in comparison to VEH-treated controls (fig. 5.2).

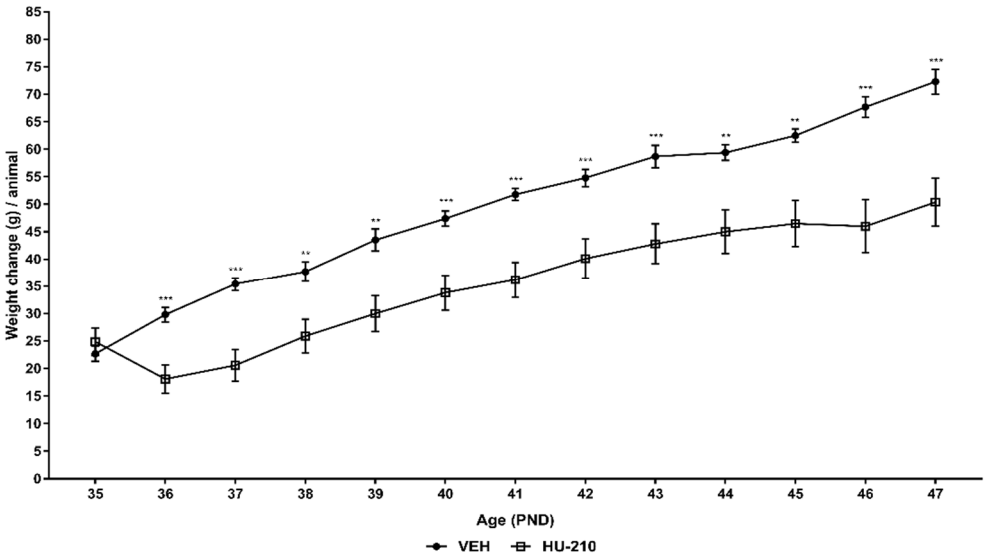

Fig. 5.2 – Change in animal weight relative to PND 28 over the course of the experiment. HU-210 treatment significantly reduced the amount of weight animals gained during the entirety of the experiment. Data are expressed as mean ± SEM (n=10); ** $p \leq .01$, *** $p \leq .001$, unpaired Student's *t*-test with Holm-Sidak correction.

3.2 – Behavioral Testing

3.2.1 – Open Field Test

Given that only one OFT session was performed, animals were simultaneously assessed for anxiety- and locomotor activity-related parameters. Regarding the former (fig 5.3a), no differences were found between groups in either the time spent (VEH = 5.48 ± 0.69, HU = 5.16 ± 0.79; t(18) = 0.32, p = .753), or distance traveled (VEH = 7.13 ± 0.6, HU-210 = 7.77 ± 0.57; t(18) = 0.776, p = .448) in the CZ. Similarly, when assessing the average velocity of the animals during the trial, no differences were found (VEH = 22.3 ± 1.13, HU-210 = 22.9 ± 1.03; t(18) = 0.395, p = .697; fig. 5.3b). However, a tendency was observed in the total distance traveled during the 10-minute trial, whereby HU-210-treated rats showed a slight, statistically non-significant, decrease in this parameter (VEH = 3931 ± 165, HU-210 = 3523 ± 107; t(17) = 2.02, p = .059; fig. 5.3c).

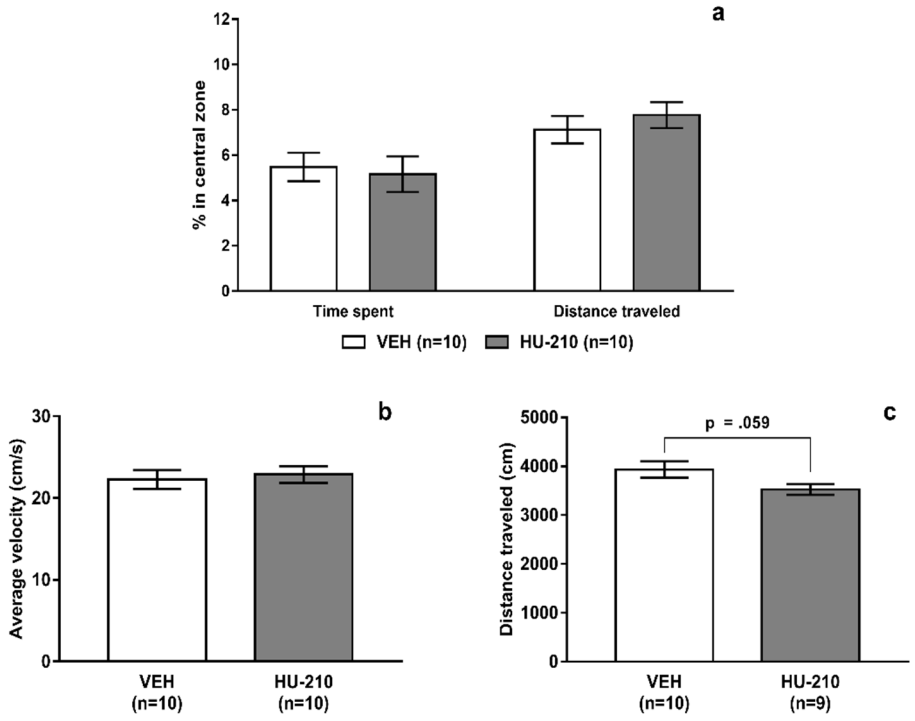

Fig. 5.3 – Chronic adolescent exposure to HU-210 did not alter anxiety-like behavior or locomotor activity in the OFT 24-hours after the last drug administration. Two measures of anxiety **(a)** were derived from the OFT: percentage of time spent and distance traveled in CZ, neither of which showed significant differences across groups. Likewise, average velocity **(b)** and total distance traveled **(c)** during the entirety of the trial did not differ between groups, despite a tendency towards a difference in the latter parameter. Data are expressed as mean ± SEM (n=9-10); unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.7).

3.2.2 – Modified Forced Swim Test

The first mFST session, lasting 15-minutes (fig. 5.4a), was performed on PND 46, immediately after the end of the OFT. Analysis revealed statistically significant differences between groups, whereby HU-

210-treated animals spent more time climbing (VEH = 104 ± 8.87, HU-210 = 264 ± 22.5; t(17) = 6.34, p ≤ .001), and less time immobile (VEH = 553 ± 17.3, HU-210 = 423 ± 26; t(17) = 4.08, p ≤ .001), than their VEH-treated counterparts, with no differences in terms of swimming behavior (VEH = 256 ± 10.2, HU-210 = 213 ± 19.8; t(17) = 1.85, p = .082).

To confirm that these results did not stem from instability in behavioral patterns, in the first mFST session, a second, 5-minute session (fig. 5.4b) was performed on the next day (PND 47). Here, the results were essentially the same: HU-210-treated animals spent significantly more time climbing (VEH = 51.2 ± 6.97, HU-210 = 144 ± 19.7; t(17) = 4.24, p ≤ .001), and significantly less time immobile (VEH = 165 ± 12.8, HU-210 = 104 ± 17.8; t(18) = 2.81, p = .012), than VEH-treated controls, without showing differences in the time spent swimming (VEH = 66.1 ± 4.72, HU-210 = 52.8 ± 7.7; t(17) = 1.43, p = .17).

Additionally, as a way to further determine whether performance in the first mFST session was the product of behavioral instability, a correlational analysis was performed for each of the behaviors, in both sessions, for each animal. This analysis revealed strong (r = 0.60 – 0.79) to very strong (r = 0.80 – 1) significant correlations, between the levels of climbing (r = 0.872, n = 19, p ≤ .001), swimming (r = 0.608, n = 18, p = .007) and immobility (r = 0.786, n = 19, p ≤ .001) in the first and second sessions.

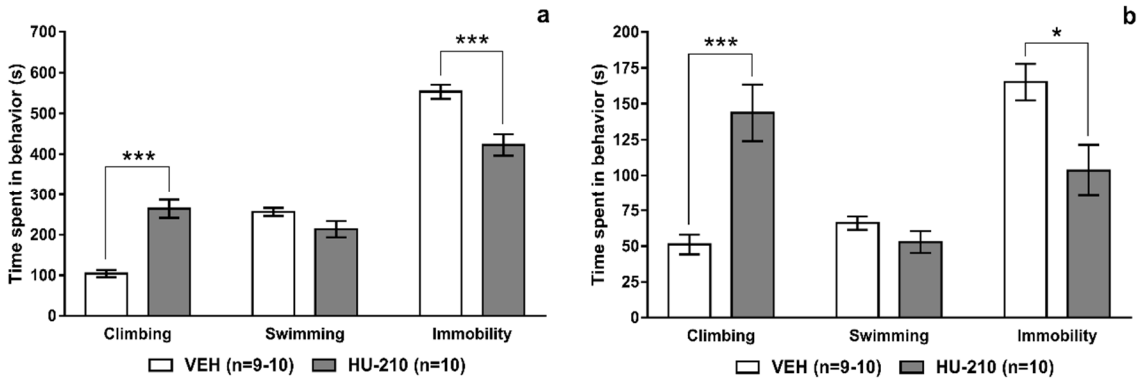

Fig. 5.4 – Chronic adolescent exposure to HU-210 altered stress-coping behavior in the mFST 24-hours after the last drug administration. Three parameters were assessed during the 15-minute **(a)** and 5-minute **(b)** mFST sessions: climbing, swimming and immobility. In both sessions HU-210-treated animals presented increased levels of climbing behavior and concomitantly decreased levels of immobility, in relation to controls. Data are expressed as mean ± SEM (n=9-10); * p < .05, *** p ≤ .001; unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.7).

3.3 – Western Blot

To investigate whether the immediate behavioral alterations resulting from HU-210 treatment were paralleled at the level of CB_1R expression, Western Blots were performed for the same three brain regions already analyzed in experiment 2.

No changes were found in the levels of CB_1R protein in either the striatum (VEH = 100 ± 8.03, HU-210 = 84.8 ± 14.2; t(7) = 0.864, p = .416; fig. 5.5b) or the PFC (VEH = 100 ± 21.8, HU-210 = 103 ± 3.33; t(7) = 0.14, p = .892; fig. 5.5c) of HU-210-treated animals. However, a marked and significant decrease was found, in the levels of this receptor, in the hippocampal tissue obtained from animals treated with HU-210 (VEH = 100 ± 3.63, HU-210 = 54.7 ± 5.61; t(8) = 6.78, p ≤ .001; fig. 5.5a).

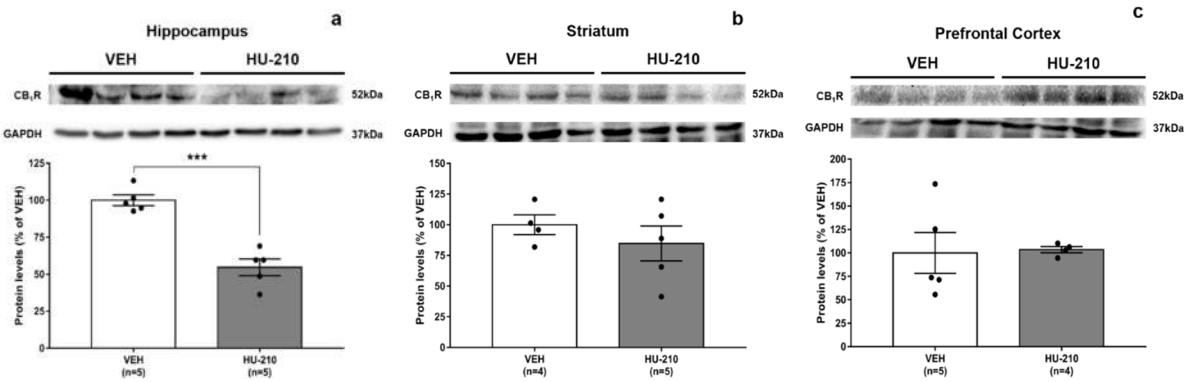

Fig. 5.5 – Chronic adolescent exposure to HU-210 selectively altered the levels of CB_1R protein, in regions involved in affective functioning, 24-hours after the last drug administration. The levels of CB_1R protein were quantified through Western Blot. Significant decreases were found in the hippocampus **(a)**, but not in the striatum **(b)** or the PFC **(c)**, of the HU-210-treated group. Data are expressed as mean ± SEM (n=4-5); *** p ≤ .001, unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.7).

4 – Discussion

The present experiment was designed with two purposes: for one, to allow the determination of whether hypothesized differences in the pharmacology of HU-210, in relation to other CBRAs, lead it to either causing effects that revert to normality after a washout period, or make it so that this drug does not behaviorally impact adolescent animals. Secondly, to further elucidate the role that age at the moment of exposure plays in the effects of this HU-210.

As was the case in both the previous experiments, HU-210 treatment induced highly significant decreases in the amount of weight the animals gained over the course of the experiment, further cementing the notion that reductions in weight-gain are a clear phenotype of HU-210 exposure.

Because only a single OFT session was performed, behavior in that session had to be analyzed for measures relating to both anxiety-like behavior and locomotor activity. While this is not ideal (see chapter 2), no changes were found in either locomotor activity-related parameter, with only a tendency for a decrease in the total distance traveled by the HU-210-treated animals – suggesting a possible hipolocomotive effect of treatment. Nevertheless, because this tendency did not reach statistical

significance, the results suggest that 24-hours after the last HU-210 injection, animals did not present treatment-induced locomotor alterations. Furthermore, groups did not significantly differ in either of the anxiety-related measures, thus suggesting that even when tested immediately after the end of drug administration, chronic adolescent HU-210 does not impact anxiety-like behavior in this test, in contrast to what has been previously observed in adult animals[493].

However, in contrast to that tendency towards decreased locomotor activity, but in line with previous work with this drug in adult animals[213,390], performance in the mFST revealed a marked *antidepressant* effect of treatment, in both sessions, characterized by highly significant increases in climbing behavior, and equally highly significant decreases in the time spent in immobility. While it could be argued that this performance was an artifact of withdrawal-induced behavioral alterations, it is unlikely that this is the case given both the likely long half-life of HU-210[377] and the fact that Morrish et al.[390], found no difference in the performance of animals undergoing withdrawal in comparison to a drug-maintained group. Furthermore, even in animals undergoing rapid rimonabant-precipitated withdrawal, mFST performance was not indicative of depressive-like behavior, but was instead brought to control levels[390]. Thus, results seemingly suggest two things: for one, it seems that HU-210 is indeed capable of inducing behavioral alterations in adolescent animals, and, as such, the lack of adult behavioral alterations found in experiment 2 is likely the product of these alterations having been normalized during washout. Secondly, it seems that HU-210 differs from other CBRAs, not just in that it does not induce long-term effects (at least at the doses used here), but also in the fact that the immediate effects that it does induce are not age-dependent[496].

These behavioral results must, however, be taken cautiously, as they were also accompanied by a concomitant decrease in hippocampal protein levels of CB_1R, in line with previous reports[294,333,334]. Indeed, these receptors are known to be down- and upregulated, by chronic stress regimens[198i], and chronic antidepressant treatment[514], respectively. Moreover, unlike it has been previously reported in several studies with other CBRAs using female animals[291,294,333], no changes were observed in the levels of either striatal or prefrontal CB_1R. However, while these disparities may be further indication of differences

[i] It should be noted that when female animals are exposed to chronic stress paradigms an increase in CB_1R density is found in hippocampal tissue[201]. While this is apparently in contradiction with the results observed here, it the fact that a potent CBRA is used in the present work may explain this difference.

between HU-210 and other CBRAs, or stem from the dissimilar techniques employed, it must also be noted that some studies have also found no differences in these regions after adolescent CBRA treatment[332,334].

Finally, finding strong significant correlations between behavior in the first and second mFST sessions, suggests that, while behavioral instability may be present to some extent in the first session, it is not enough to substantially bias the behavioral outcomes observed in both the first two experiments, as well as in the literature. That is, while behavioral instability may have had some impact on the discrepant results observed in experiment 2, it is unlikely that those results were a full artifact of it.

While in accordance with the work previously published using adult rats, the present results are still somewhat counterintuitive, given the known pro-depressant actions of prolonged exposure to other CBRAs. This is especially more relevant when the sole measure of locomotor activity used was the OFT – whose reliability in measuring this parameter is, at best, controversial[393,426]. Indeed, from observation and manipulation of HU-210-treated animals in this experiment, it was quite evident that treatment induced marked alterations. Specifically, much in line with previous reports, animals were noticeably hyperactive, reactive and aggressive[383,385,387]. As such, and in line with the – as of yet untested by anyone – suggestion laid out by Morrish et al.[390], a fourth experiment was designed to assess another facet of depressive-like behavior, using a test that is independent of locomotor activity.

Chapter 6 – Experiment 4

1 – Rationale

While the results obtained in experiment 3 are in line with previous reports with adult animals chronically exposed to HU-210[390], it is still unexpected that prolonged exposure to a highly potent CBRA would lead to such a marked antidepressant-like effect – especially in adolescent animals[496]. Moreover, given that the sole measure of locomotor activity used in experiment 3 was the OFT – a test whose reliability for measurement of locomotor activity has come into question[393,426] – a possibility remains that the mFST results may have somehow be an artifact of locomotor effects of treatment. Indeed, in the discussion of their work, Morrish et al. suggest that complementary tests of depressive-like behavior should be performed, in order to further confirm these results[390]. This suggestion has not, however, yet been pursued in any published research.

As such, the present experiment was performed with the intent of exploring the immediate effects of chronic adolescent exposure to HU-210, on another dimension of affective functioning – that of reward functioning – through the use of the SPT, a test that is independent of locomotor activity. Additionally, to further complement the results obtained with the OFT, and construct a clearer picture of how this treatment impacts anxiety-like behavior, animals were also tested in the EPM.

2 – Methods

2.1 – Animals and Ethical Approval

Twenty female Sprague-Dawley rats, aged 21 days (PND 21) at the time of arrival, were ordered from Charles River Laboratories (Calco, Italy) and were housed in groups of five, in the same conditions as described in experiment 1. A period of at least three days of acclimatization was allowed before any experimental procedure was performed, and animals were monitored daily for physical and behavioral signs of distress and/or suffering.

All experiments took place during the light phase of the cycle, and were performed in conformity with European Community Guidelines (Directive 2010/63/UE), and with the approval of the Committee for

Ethics in Animal Research of the Faculty of Medicine of the University of Lisbon, as well as of the Portuguese Competent Authority for Animal Welfare.

2.2 – Drugs

The same HU-210 stock solution at 25mM concentration used in experiment 2 was used here. From this stock solution further dilutions were made each day, in 0.9% saline, to reach adequate volume.

2.3 – Drug Administration

At the time of arrival animals were randomly assigned to be treated with either HU-210 (n=10) or vehicle solution (VEH; n=10).

Drug administration (fig. 6.1) was performed in every way similarly to that described for experiments 2 and 3.

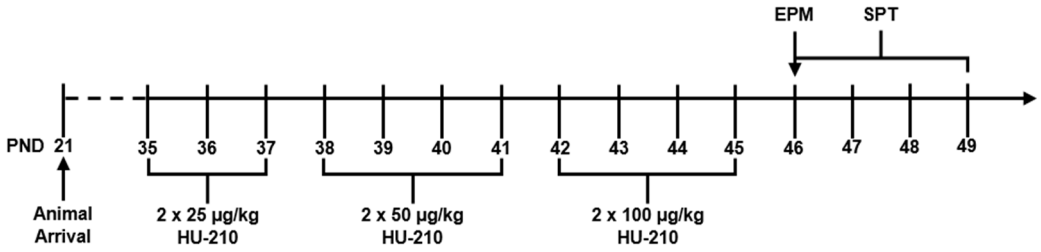

Fig. 6.1 – Chronogram of drug administration and behavioral testing. EPM, elevated plus maze; SPT, sucrose preference test.

2.4 – Animal Body Weight

Animal body weight was monitored during the entirety of the experiment, with animals being weighed daily for the full period of drug administration and behavioral testing (PND 35-49).

To calculate weight changes, the starting body weight of each animal (PND 28) was subtracted to its body weight at each time-point, with the resulting values (expressed in grams) being used to compare the effects of treatment across groups.

2.5 - Behavioral Testing

The day after drug administration period ended (PND 46), animals were subjected to behavioral testing, so as to further assess the immediate effects of chronic HU-210 exposure during adolescence. Two tests were performed: the EPM, as a measure of anxiety-like behavior and locomotor activity, and the SPT to assess reward functioning (fig. 6.1). To reduce animal stress, and its possible confounding effect on

behavioral performance, rats were individually handled for at least five minutes, on the five days preceding the first test (PND 41-45), after morning injections.

Behavioral testing and analysis procedures were in every way similar to those described in experiment 1.

2.5.1 – Elevated Plus Maze

The EPM was performed and scored similarly to that described in experiments 1 and 2, with the sole difference being that testing occurred on PND 46. Furthermore, the total number of zone changes during the 5-minute trial, was derived from this test, as a measure of locomotor activity.

2.5.2 – Sucrose Preference Test

The SPT was performed and scored similarly to that described in experiments 1 and 2, with the sole difference being that the testing occurred from PND 46 to PND 49.

2.6 – Statistical Analysis

Statistical analysis was in every way similar to that described in experiments 1, 2 and 3. All data are expressed as means ± SEM, or as medians and IQR, or range (minimum and maximum values, wherever appropriate), depending on whether parametric or non-parametric tests were used, respectively. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

3 – Results

3.1 – Animal Body Weight

Similarly to what was done on experiments 2 and 3, animals were weighed on PND 28, with no difference being observed between groups (VEH = 97.4 ± 1.96, HU-210 = 97.6 ± 1.56; $t(18) = 0.8$, $p = .937$). When changes in weight from this baseline were assessed, no effect was detectable on the first day of drug administration (PND 35). However, on the second day of treatment, a difference emerged, whereby HU-210-treated animals gained significantly less weight than their VEH-treated counterparts (fig. 6.2). This effect remained significant for the remainder of the experiment (all comparisons at least $p < .05$).

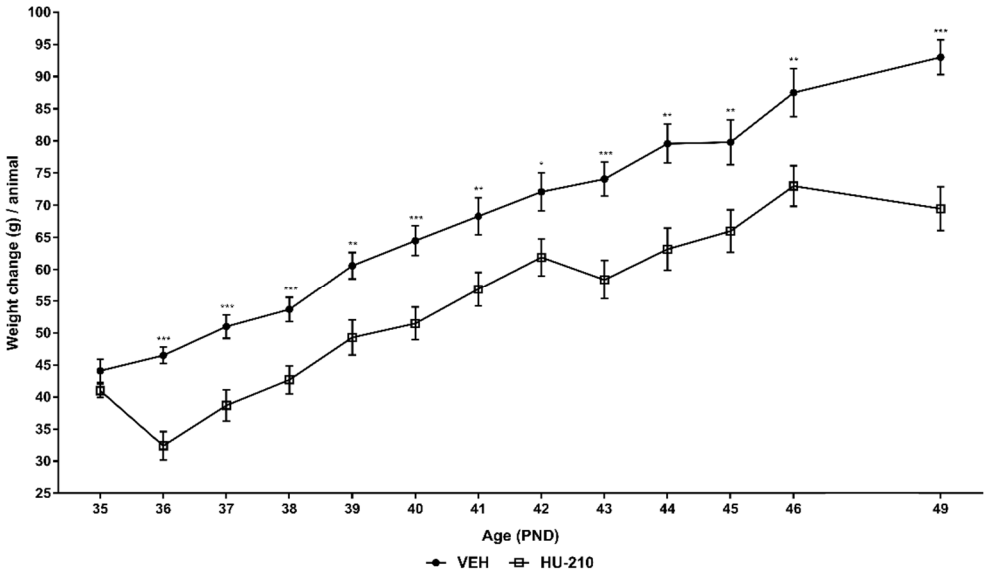

Fig. 6.2 – Change in animal weight relative to PND 28 over the course of the experiment. HU-210 treatment significantly reduced the amount of weight animals gained during the entirety of the experiment. Data are expressed as mean ± SEM (n=10); * $p < .05$, ** $p \leq .01$, *** $p \leq .001$, unpaired Student's *t*-test with Holm-Sidak correction.

3.2 – Behavioral Testing

3.2.1 – Elevated Plus Maze

To expand on the immediate effects of chronic adolescent exposure to HU-210, on anxiety-related behavior, the EPM was performed the day after drug administration ceased.

Analysis of the anxiety-related parameters showed that HU-210-treated animals did not significantly differ from controls regarding either the percentage of time spent (VEH = 32.2 ± 3.62, HU-210 = 40.3 ± 5.06; $t(16) = 1.35$, $p = .196$; fig. 6.3a), or in the number of entries in the open arms (VEH = 11.3 ± 1.41, HU-210 = 13.5 ± 0.73; $t(16) = 1.28$, $p = .219$; fig. 6.3b). Furthermore, because EPM performance may be biased by locomotor alterations, the total number of zone changes was taken as an index of locomotor activity, with no differences between groups being detected regarding this parameter (VEH = 44.9 ± 2.16, HU-210 = 50 ± 2.38; $t(15) = 1.6$, $p = .131$; fig. 6.3c).

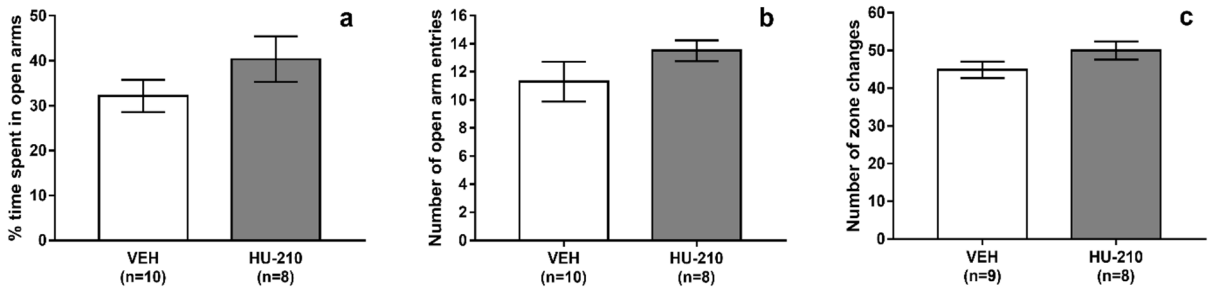

Fig. 6.3 – Chronic adolescent exposure to HU-210 did not alter anxiety-like behavior in the EPM 24-hours after the last drug administration. The EPM was performed on PND 46 with two measures of anxiety being derived from the 5-minute trial, neither of which showed significant differences: percentage of time spent in the open arms **(a)**, and the total number of open arm entries **(b)**. Additionally, the total number of zone changes was used as an index of locomotor activity **(c)**, with no differences being found for this parameter. Data are expressed as means ± SEM (n=8-10); unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.6).

3.2.2 – Sucrose Preference Test

To assess if, like stress-coping, reward functioning (another facet of affective functioning), is also altered immediately after the end of chronic adolescent HU-210 exposure, the SPT was performed from PND 46 to PND 49.

Analysis of sucrose intake revealed a marked effect of HU-210 treatment, whereby HU-210-treated animals consumed significantly less sucrose solution than the VEH-treated animals in both the first (VEH = 0.29 ± 0.04, HU-210 = 0.15 ± 0.23; $t(17) = 3.14$, $p = .006$), second (VEH = 0.39 ± 0.07, HU-210 = 0.13 ± 0.03; $t(17) = 3.72$, $p = .002$) and third (VEH = 0.64 ± 0.15, HU-210 = 0.17 ± 0.02; $t(17) = 2.91$, $p = .01$) days of the test (fig 6.4a). Moreover, this difference also extended to the average daily intake over the duration of the SPT (VEH = 0.5 ± 0.1, HU-210 = 0.16 ± 0.03; $t(18) = 3.25$, $p = .004$; fig. 6.4b).

Likewise, analysis of relative preference revealed a similar effect: HU-210-treated rats showed markedly inferior sucrose preference in comparison to controls at both the first (VEH = 85.4 ± 2.39, HU-210 = 59.7 ± 7.07; $t(17) = 3.29$, $p = .004$), second (VEH = 82.1 ± 2.59, HU-210 = 44 ± 6.14; $t(17) = 5.49$, $p \leq .001$) and third (VEH = 84.9 ± 2.36, HU-210 = 53.4 ± 5.64; $t(17) = 4.95$, $p \leq .001$) measurement moments (fig 6.4c). Thus, at all time-points HU-210-treated animals met the criteria for being anhedonic (i.e., preference < 60%). Furthermore, average preference over the duration of the test was also found to be significantly different between groups, with HU-210-treated rats showing diminished preference for sucrose (VEH = 84.4 ± 2.41, HU-210 = 52.4 ± 5.15; $t(17) = 5.43$, $p \leq .001$; fig 6.4d).

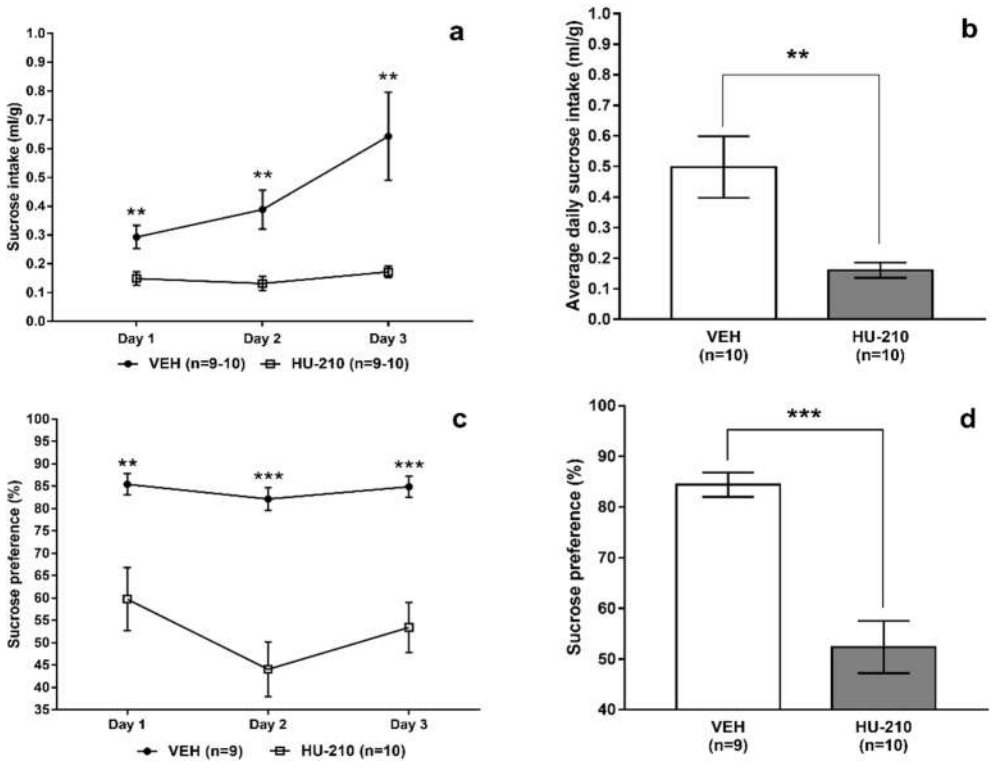

Fig. 6.4 – Chronic adolescent exposure to HU-210 altered sucrose intake and preference 24 to 96 hours after the last drug administration. Sucrose intake was assessed daily **(a)** and was averaged at the end of the testing period **(b)**, with HU-210-treated animals presenting marked decreases in both parameters. Similarly, sucrose preference was severely decreased in both daily assessments **(c)**, and when averaged over the entirety of the test **(d)**. Data are expressed as mean ± SEM (n=9-10); ** $p \leq .01$, *** $p \leq .001$, unpaired Student's *t*-test. Reported group sizes exclude significant outliers (see section 2.6).

4 – Discussion

The present experiment was designed to further elucidate and expand the surprising results obtained in experiment 3, by testing a new batch of animals in two other tests of anxiety-like (EPM) and depressive-like (SPT) behavior, immediately after the end of prolonged HU-210 administration. The results obtained were, however, highly unexpected.

As it was found in the first three experiments, in the present experiment, HU-210 treatment was also found to lead to decreased weight-gain. This decrease was significant on the day following first drug injections, and remained so for the duration of the experiment.

Regarding the EPM, no differences between groups were found in either anxiety-like behavior or locomotor activity, in line with the results obtained for the OFT in experiment 3. Thus, these results further support the assertion that, even though HU-210 is capable of inducing marked effects on affective functioning, these are restricted to depressive-like behavior, without affecting anxiety-like behavior.

In surprising contrast to the antidepressant-like effect observed in the mFST in both experiment 3 and adult studies[213,390], in the present experiment HU-210-treated rats presented a marked depressive-like phenotype in the SPT: in comparison with VEH-treated controls, the HU-210 group showed highly significant decreases in both sucrose intake and preference, at all time-points, as well as when considering the average of the three-day period, indicating a strong anhedonic effect of treatment.

An explanation for these, apparently contradictory, results is not immediately self-evident, but two possibilities stand out as the most likely: on the one hand it is possible that chronic HU-210 – at least in adolescent animals – induces opposite effects on different aspects of affective functioning. On the other hand, it may be the case that either the results obtained here, or in experiment 3, are false positives. If the results presented in this experiment were to be false positives, a likely source of this outcome would be the effects of cannabinoid-withdrawal. However, this seems unlikely, given that if this were to be the case one would expect a progressive change in SPT parameter, as withdrawal symptoms evolved (i.e., improved or worsened) over the three-day period – something that was not observed in HU-210-treated animals (fig 6.4). Thus, it seems more likely that, if a false positive does exist, it would be the results from the mFST in experiment 3. Indeed, in that experiment, molecular analysis was more congruent with a prodepressant-like effect[198], than with an antidepressant-like effect[514]. Moreover, not only is the mFST susceptible to being biased by alterations of locomotor activity[450,461], but it has also been shown that this test may provide inconsistent results when it comes to manipulations of the ECS. Specifically, despite its known *prodepressant* effect in humans[31,202], the CB_1R antagonist/inverse agonist rimonabant has been inconsistently been identified as prodepressant in this test – with some studies even suggesting that it might have antidepressant effects[515,516].

Considering the results of all the experiments thus far presented, numerous questions remain as to why and how HU-210 induces the unexpected and contradictory behavioral outcomes observed, an issue discussed in detail in the next section.

Chapter 7 – General Discussion, Future Perspectives, and Conclusions

The lasting effects of chronic adolescent exposure to CBRAs on adult affective functioning have been amply characterized. However, the overwhelming majority of the research done so far has relied on a small pool of drugs, leaving open the possibility that the effects observed are only a part of the full spectrum of possible outcomes. The present work, by focusing on a drug not yet tested with regards to this dimension of its possible effects, aimed to help begin filling that gap. Interestingly, the results obtained suggest that the overreliance on a select group of CBRAs may not be desirable, given that the outcomes observed here were markedly different from those expected at the outset, based on previous studies.

The present chapter constitutes a brief overview of the results obtained across the 4 experiments performed, and includes a number of possible explanations for them. Furthermore, in the final section of this chapter, a discussion will be made with regard to future work, that should be performed to test the veracity of the proposed explanations.

1 – General Discussion

1.1 – Body Weight Changes

The most consistent result obtained, across the four experiments here performed, relates to the effects of HU-210 on the changes in body weight. Specifically, in all four experiments, animals showed highly significant decreases in weight gain, relative to an original measurement (PND 35 in experiment 1, and PND 28 in experiments 2-4), which were already evident on the day following first administration. This was the case even though no such differences were observed in the first day of drug administration. Moreover, this was also observed in experiment 1, despite the fact that – by chance – the HU-210-assigned group weighed significantly more at the experiment outset. Furthermore, in all experiments, this decrease in weight gain persisted (and remained highly significant) for the entirety of the drug administration period, and – in the case of the first two experiments – lasted for 15 days after the last injection.

At first glance this result would seem to be in marked contradiction with both the anecdotally reported orexigenic effects of cannabis, as well as the published reports testing the veracity of these anecdotal reports[501,502]. However, it should be noted that, in human users, cannabis use has been linked to a decrease

in body mass index[517]. Moreover, decreased food intake, body weight and weight gain have been consistently reported in rodent studies ever since the 1970s[503,518,519]. Indeed, a study by Giuliani et al.[520], also found chronic HU-210 exposure to lead to significant lasting decreases in body weight and food intake.

Several explanations have been proposed for this orexigenic effect of CBRAs. For one, it is possible that the locomotion-impairing effects of these drugs may make animals less able to reach food, thus decreasing food intake[294]. This, however, seems unlikely given that not only does tolerance to the locomotor effects of CBRAs develop fairly rapidly[384,388], but also that, in the first two experiments, the decreased weight gain persisted for an extended period after drug administration, where it is improbable that significant drug concentrations would still remain in the organism of the animals.

Secondly, there is the possibility that CBRAs selectively alter feeding behavior, such that they bias the animals towards a specific type of food, in detriment of others. Indeed, it has been shown that acute CBRA administration selectively increases intake of high fat or sweetened foods, without altering standard rat chow intake[521]. Since the animals used here were fed a standard diet, this may possibly explain, or contribute to, the decreases in weight gain.

Thirdly, there is a possibility that CBRAs may work to modulate the rewarding properties of food, through their interactions with the opioid[522] and/or DA systems[523]. In line with this hypothesis, alterations have been found in both of these systems in animals chronically exposed to CBRAs as adolescents[223,264,278,280,292,294,306,319,320,325–327], and alterations in the intake of palatable foods and/or sucrose solutions have been reported[210,276,294]. While these decreases in the intake of palatable/sweet foods are in disagreement with the previously proposed explanation (i.e., a preference for sweetened foods), this hypothesis would fit with the findings of markedly decreased sucrose solution/preference found in experiment 4. Moreover, this explanation would also be consistent with the lack of effects found, in the SPT, in the first two experiments. In other words, as weight gain is normalized after 15 days without drug exposure, so may the reward and motivation circuitry of HU-210 exposed rats. Accordingly, Bellocchio et al.[524], showed that CBRAs have a biphasic effect on feeding behavior, with low doses leading to hiperphagia and moderate to high doses to hypophagia, and that this effect is contingent on whether glutamatergic or GABAergic ventral striatal neurons are inhibited by CB_1R activation, respectively. Were this hypothesis to hold true, a question would still remain as to why a less potent and less effective CBRA,

such as THC, led to lasting DAergic alterations – such as those reported by Renard et al.[278,306] – while HU-210, apparently, had no such effect.

Another possible explanation is that chronic CBRA exposure may lead to downregulation and/or desensitization of hypothalamic CB_1R. Indeed, this brain region is well-known to be involved in the regulation of numerous homeostatic processes[525], including feeding[526], and there is an amply described interaction between hypothalamic ECS and orexic signaling[527]. As such, it has been proposed that hypothalamic CB_1R downregulation may disrupt the capacity of the ECS to effectively stimulate orexigenic signaling[528]. This would, in essence, represent a functionally similar effect to that of the CB_1R antagonist/inverse agonist rimonabant, which is known to be anorexigenic. Importantly, this hypothesis is not mutually exclusive with that postulating a role for the mesolimbic reward system, since it leaves open the possibility of a concomitant pro-amotivational effect of CBRAs. Critically, however, it is unlikely that, in short time-scales, the anorexigenic effects of HU-210 would be caused by hypothalamic CB_1R downregulation, since these effect are observable within 24-hours of treatment – when downregulation is unlikely to have yet occurred. It may, nonetheless, be the case that, in the earlier stages of drug administration, it is the acute hypolocomotor effect of HU-210 that induces the decreases in weight gain, whereas, at later time-points, this anorexigenic effect results from hypothalamic CB_1R downregulation.

Finally, the effect of CBRAs on hypothalamic signaling are also key part of another possible explanation for the pattern of decreased weight gain observed here. Specifically, it has been proposed that HU-210 may induce these changes by altering adequate HPA axis functioning[520]. Indeed, HU-210 is known to lead to marked increases in the secretion of CORT, ACTH and CRF[373,520], which are known to negatively modulate food intake[529–531]. Moreover, the effects of prolonged increases in stress-hormones are known to be persistent[532–534], which may explain the persistence of significantly decreased weight gain in the 15 days following drug administration.

1.2 – Anxiety-like Behavior, Social Anxiety-like Behavior, and Locomotor Activity

Despite the fact that, in epidemiological studies, adults who used cannabis as adolescents are found to be at an increased risk of developing anxiety disorders (especially if they are females[254,255]), in the present work no such effect was found. Indeed, independently of whether animals were tested immediately after

drug administration ended, or after a washout period, no evidence of altered performance was observed in either the EPM, the OFT or the MBT. Nonetheless, despite this contradiction with human data, these results were not entirely unexpected, given that, although some animal studies do find chronic adolescent CBRA exposure to lead to lasting alterations in anxiety-like behavior[271,289,295,296,299–301,304,305], the majority of studies have not found such effects.

The reasons for this lack of effect are not entirely clear. Indeed, when CBRAs are acutely administered to rodents, they commonly alter anxiety-like behavior in a biphasic manner, such that low doses have an anxiolytic effect, whereas high doses lead to anxiogenic effects[535]. Moreover, in one study using adult rats[493], chronic exposure to the highest dose of HU-210 used here (100 μg/kg) led to decreased time spent in the OFT CZ – suggesting a anxiogenic effect of drug exposure – when testing was performed on the day following last drug administration. Critically, however, these same animals showed no evidence of altered performance in the EPM[493]. Contrastingly, using a similar drug administration schedule, Jiang et al.[213] found an anxiolytic effect in the NSFT, after a 30-day washout.

Several explanations may be posed for this virtual lack of effects upon anxiety-like behavior: for one, it may be the case that adolescent animals are less vulnerable to the anxiety-modulating effects of CBRAs, than adults. Indeed, Carvalho et al.[336] reported an age-related difference in the aversive properties of sub-chronic high-dose WIN 55,212-2 administration, such that adolescent animals were shown to be less sensitive to these effects, than adults. Given that aversive stimuli reliably alter performance in tests of anxiety-like behavior[536–538], this may explain the discrepancy between results found here and those obtained with the same drug in adult animals. This explanation is further supported by the fact that there is significant difference, between adult and adolescent animals, with respect to how fast such tolerance develops[497]. Thus, it is conceivable that the adult animals in work the wok of Hill and Gorzalka[493] had not yet developed full tolerance to the anxiogenic effects of high-dose HU-210, whereas the animals in the present experiment may have done so. Indeed, this may have happened despite our best efforts to preclude that from happening, through the use of an escalating dosing schedule. A limitation of this explanation, however, lies in that it leaves unanswered the question of why similar exposure to HU-210 led, in the study by Jiang et al. [213], to an anxiolytic-like effect, after a 30-day washout period.

Alternatively, it may be that we (and previous groups) have found no effects on anxiety-like behavior not because chronic adolescent CBRA exposure does not induce such alterations, but because the assessment of anxiety-like behavior is not adequate. That is, because the behavioral tests employed here have limited predictive validity[420,421,424] (see chapter 2), it may be that they are unable to detect alterations in anxiety-like behavior, induced by adolescent CBRA treatment. Indeed, while the EPM and OFT have been widely used, these tests seem grossly inadequate in the detection of pharmacological manipulations of anxiety, and some researchers have even questioned whether these assays can be considered to be modelling pathological anxiety[415,424,438,539]. Thus, the discrepancy between epidemiological data and animal behavioral data may stem from this limitation in the testing used in animal research.

The above explanation suffers, however, from two problems: firstly, while the EPM and OFT have the aforementioned limitation in detecting pharmacological manipulations of anxiety-like behavior, this has not – thus far – been the case for the MBT[420,421]. However, no alterations were found in this test either, leading to one of two conclusions: either the MBT is also inadequate to detect this type of manipulation, or chronic adolescent CBRA exposure does not lead to long-term alterations of anxiety-like behavior in animals, despite doing so in humans[254,255]. Irrespective of which of these conclusions may turn out to be correct, it must be noted that the use of the MBT in this work, is – to the best of our knowledge – the first time that this test has been used in cannabinoid research. As such, this represents a truly novel finding. Secondly, a question remains as to why some studies, using the EPM and the OFT as measures of anxiety-like behavior, do find some persistent alterations[271,289,295,296,299–301,304,305], whereas here (and in most other studies) no such differences are found. While no ready explanation can be suggested for this discrepancy, a careful consideration must be made as to a possible reason for it: of the few studies that have found adult anxiety-like behavior to be either increased or decreased as a consequence of adolescent CBRA exposure, none have used the same combination of animal strain and gender used here[271,289,295,296,299–301,304,305]. Indeed, no study using Sprague-Dawley rats has found anxiety to be altered as a consequence of chronic adolescent CBRA exposure, and of the few that have used Wistar rats (such as those used in experiment 1), all but two[271,300] have exclusively used males[299,301,304]. Furthermore, given that this is the first study testing the effects of adolescent HU-210 exposure on anxiety-like behavior, there is a possibility that the disparity

between the results presented here, and those of the few studies where alterations were found in this parameter, stems from the difference in drug used.

While the differences in general anxiety-like behavior are largely in agreement with the previous literature, the lack of observable effect in social interaction is not. Indeed, decreased social interaction has been one of the most consistently reported consequences of chronic adolescent CBRA exposure, having been observed in studies using both Wistar and Sprague-Dawley rats, as well as with several CBRAs[261,262,273,274,276,282,308,506]. However, here, in both experiments 1 and 2, no such effect was found. Importantly, with the exception of the works by Zamberletti et al.[268,280], the two other works where no alterations were found in adult social behavior, used mice[318], or rats from a different strain[264].

Given the known widespread involvement of the ECS in the regulation of social behavior[540,541], this lack of effect is difficult to explain. The most obvious explanation is that, as seems to be the case with depressive-like behavior, any alteration that may have been induced by chronic adolescent exposure to HU-210, was normalized during the washout period. Indeed, even at the molecular level, alterations induced by treatment were found to be absent, when rats were given a 30-day drug washout (i.e., hippocampal CB_1R protein levels were found to be reduced soon after the end of drug exposure, but to not be altered at adulthood). Moreover, if one were to give primacy to the reward-component of the SIT[310], then this result would also fit with the lack of alterations in the SPT.

This suggestion, however, would depend on the notion that HU-210 has pharmacological properties that are not shared by other CBRAs. This notion still requires confirmation, despite being suggested by other results herein reported, as well as by previous molecular pharmacology work[293]. For example, it could be the case that, given the interactions between HU-210 and glycine receptor subunits (not shared with THC[379,380]), HU-210 has less impact on the social dimension of anxiety, than other CBRAs. Indeed, in line with this drugs targeting the glycine receptor have been shown to modulate anxiety-like behavior[542].

Another possibility is that the testing conditions in which the SIT was performed may have precluded the detection of any effects on anxiety. Specifically, while in the present work animals were familiarized with the arena over two 10-minute sessions[443,444], with light kept at a constant intermediate level, there is evidence that the anxiety levels induced by this test may be contingent on factors such as familiarity with the testing apparatus, or light levels[443,444]. Thus, a possibility exists that inadvertent differences between

the environment in which the tests were performed in the experiments here described, and those in which other groups performed their tests, explain the differences in outcome.

Finally, it must be noted that in none of the experiments was the spontaneous locomotor activity of HU-210-treated rats found to be altered. This result should, however, not be taken at face value. As discussed in chapter 2, OFT measures of locomotor activity are likely not reliable indexes of this parameter[426], and the use of EPM-derived measures (experiment 4) is even less so[414]. This lack of locomotor alterations was not, however, completely unexpected, as CBRA induced changes in locomotor activity seem to be relatively restricted to cases of acute administration[382–384], or during the initial stages of chronic exposure, with tolerance developing fairly rapidly[357] (but see[278,306]). Thus, irrespective of the necessity of taking into account the questionable reliability of measures obtained with the OFT, and in the absence of any adequate substitute for these measures, the results obtained here suggest that chronic adolescent HU-210 exposure does not alter spontaneous locomotor activity in either the short-, or the long-term, after stopping administration. In light of this, any changes observed in other tests, must be considered to be relatively unaffected by biases stemming from alterations at the level of locomotor activity.

1.3 – Depressive-like Behavior

Alterations at the level of depressive-like behavior have been consistently reported in the literature concerning the long-term effects of chronic adolescent CBRA exposure[210,276,280,282,294,308,315]. Indeed, most studies find that this type of exposure leads to lasting deficits in this dimension of affective functioning, and that these deficits are more pronounced in female than male animals[359] – in line with the findings of human epidemiological data[254–256,258]. In the current work, however, the results obtained are in stark disagreement with that previously reported with other CBRAs.

Specifically, while the first experiment indicated a tendency for impaired stress-coping behavior, this finding was not replicated in the second experiment, despite the fact that, in this latter experiment, animals were exposed to a greater absolute amount of HU-210. While it is possible that this difference stems from differences in the duration of administration (and not the dose administered), or in the timing of behavioral testing, a more likely culprit is the possible deleterious effect of the solvent used as a drug vehicle. Indeed, as discussed previously, DMSO does have neurotoxic effects[507–510], that may have been more pronounced

due to the age of the animals at the time of administration. Notwithstanding, it must be noted that, even in these conditions, in experiment 1, no alterations were found at the level of reward functioning. Moreover, this was the same outcome observed in experiment 2. Thus, it seems that chronic adolescent HU-210 exposure does not induce long-lasting alterations at the level of depressive-like behavior.

This finding was entirely unexpected, given the previous literature, and the much increased potency, affinity and efficacy of HU-210, in relation to previously studied CBRAs. To further understand these findings, experiment 3 was performed to assess whether this lack of long-term effects was the result of HU-210 being unable to modulate affective behavior or, alternatively, a result of normalization of any previous effect, during the washout period. Curiously, when animals were tested immediately after stopping administration, a marked antidepressant-like effect was found – as indicated by greatly increased climbing time, and equally greatly decreased immobility time in the mFST. These findings were in line with previous studies in adult rats[213,390], but required confirmation (i.e., experiment 4) for several reasons: for one, the effects of CBRAs are known to be age-dependent[496] (and generally more negative in adolescents). Secondly, western blotting revealed a change in hippocampal CB_1R protein levels that was similar to that observed in CMS-exposed rats[198]. Thirdly, despite no alteration having been found in the OFT, personal observation/manipulation of the animals suggested the presence of strong behavioral alterations. Specifically, in line with previous reports[383,385,387], HU-210 treated animals were remarkably more reactive, aggressive and behaviorally hyperactive. Thus, experiment 4 was performed to provide a locomotor activity-independent measure of depressive-like behavior, in the form of the SPT. Notably, in this final experiment, results were entirely contrasting with those of experiment 3, with animals showing markedly impaired reward functioning, as indicated by decreased sucrose intake and preference.

As such, in combination, the results of experiments 2-4 suggest that HU-210 is indeed capable of strongly modulating the expression of depressive-like behaviors, but that these effects are relatively short-lived. Such a result, while positive for the field of cannabinoid-based therapeutics, does, however, require further consideration, since no explanation for it is immediately apparent. The most obvious explanation is that HU-210 differs from other CBRAs with regards to either its interaction with the ECS, or with other non-ECS targets. In terms of the former, it may be that the greater relative affinity of HU-210 for CB_1R over CB_2R, may be underpinning this differential effect. Alternatively, it may also be that HU-210 has a

unique profile of biased agonism, leading to the activation of a group of G-protein subtypes distinct from other CBRAs[391], triggering specific signaling pathways. If, however, the latter possibility holds true, and the source of the disparate outcomes observed lies in non-ECS interactions, there is a great number of possible targets to be considered[293]. Indeed, to the best of our knowledge, no large scale receptor binding profile has been performed, to determine to what other receptors HU-210 binds. This is even more interesting when one considers that a simple mirroring of this molecule leads to markedly different pharmacodynamics: indeed, not only does HU-211, the enantiomer of HU-210, not interact with the ECS, but it is actually a NMDAR antagonist, with known anticonvulsant and neuroprotective effects[543,544].

Similarly, with regards to the contrasting results of experiments 3 and 4, an explanation is also not self-evident. One possibility deserving future study entails the consideration of the actions that CBRAs have on both the NA and HPA axis systems. Specifically, the increased mFST climbing observed in experiment 3, and in previous adult work[390], suggest an involvement of the NA system – given that increases in this behavior are known to result from treatment with NA targeting antidepressant drugs[448,461]. Moreover, in some of the aforementioned work, this effect was shown to be attenuated or abolished by administration of α1 and β adrenergic receptor antagonists, respectively[390]. Critically, however, increased NA levels do not necessarily imply an antidepressant response. In fact, increased levels of this neurotransmitter have been found to not only mediate (at least in part) the aversive effects of CBRAs[336,545–547], but also to be increased in the cerebral spinal fluid (CSF) and excretions of human depressive patients[548]. Furthermore, it has been demonstrated that NA signaling contributes to the effects of CBRAs on HPA axis functioning[549]. Specifically, administration of α1 and β adrenergic receptor antagonists, to adult rats chronically exposed to high dose HU-210, was shown to attenuate the abnormally high corticosterone response to restraint stress[549]. In addition, CBRAs themselves modulate the HPA axis[550–553], leading to increases in the levels of stress hormones[373,382] – that underlie many of the negative effects of acute CBRA administration[383]. Conversely, the HPA axis itself reciprocally influences the release of NA[554–556]. Thus, it may be the case that chronic HU-210 exposure led to a vicious circle: initial HU-210 administration increases both HPA and NA activity[210], which further potentiate one another. These increases would be maintained by continuous drug exposure which, due to the aversive effects of high dose CBRAs[336,545,546], is itself stressful to animals.

Relatedly, given that the hippocampus – especially its most ventral portions[557], and the vSub in particular[558] – is heavily involved in the modulation of HPA axis activity, a role for this structure could also be postulated. Specifically, HU-210-induced alterations in hippocampal functioning (as suggested by decreased hippocampal CB_1R protein levels) could have led to a further aggravation of the hypothesized vicious circle. Importantly, it must be noted that, while commonly associated with negative regulation of the HPA axis, disrupted vSub functioning has also been implicated in the potentiation of the response of this system to specific types of stress[558–561], such as exposure to novel spaces, or forced restraint – both of which share some similarities to the mFST testing situation.

Dovetailing into this, the effects of disrupted HPA axis functioning on reward functioning have been fairly well described. Indeed, both environmental[562–564] and pharmacological[565,566] manipulations known to induce dysfunction in HPA axis functioning have been described to induce depressive-like alterations in the SPT, such as the ones observed in experiment 4. Furthermore, the hippocampus (especially its ventral portion) is known to have an important modulatory role over the mesolimbic reward system[557]. Thus, the alterations observed in hippocampal CB_1R protein levels, may also suggest a role for dysfunctional hippocampal activity, in the adequate regulation of reward functioning. In addition, an increase in NA levels could further add to this deleterious effect of HU-210 on SPT performance, given that NA is known to have appetite-suppressive properties during stress exposure[567,568], which could also account for the effects of HU-210 on weight-gain.

In combination, these data can be taken as the basis for an explanation of why results in experiment 3 suggest a markedly antidepressant-like effect, whereas results in experiment 4 suggest the exact opposite: that is, increased climbing in the former experiment may be a misleading effect of an actual prodepressant impact of HU-210 exposure. Specifically, the NA-release stimulatory effects of HU-210, in combination with its potentiating effects over HPA axis activity, and the interplay between the NA and HPA axis systems, may have led to exaggerated behavioral reactivity in the mFST testing situation. Indeed, such behavioral reactivity was readily observable during animal manipulation, and could conceivably be misidentified as an antidepressant-like effect, as would be the case for amphetamine-treated hyperactive animals[425]. Conversely, when animals were exposed to a testing situation where behavioral reactivity had little possibility of biasing results (i.e., the SPT, in experiment 4) the actual, prodepressant-like, effect of

chronic adolescent HU-210 exposure, became readily detectable. Critically, it is unlikely that the observations made here are manifestations of cannabinoid withdrawal, given that Morrish et al.[390] showed the altered mFST behavior to not be different between HU-210-maintained and HU-210-withdrawn rats, with differences only being evident in the group where rapid withdrawal was propitiated by administration of a CB_1R antagonist. Interestingly, despite being counter to the way the mFST is typically interpreted, the findings herein reported would actually be fairly similar to human cases of depressive disorder, where individuals present increases in restlessness and agitation, as well as in irritability, while also presenting anhedonic symptoms[314].

This explanation suffers, however, from two important limitations: for one, it leaves open the question of why this mechanism did not have a measurable impact on anxiety-like behavior, if it was in fact the cause of the alterations observed at the level of depressive-like behavior. Indeed, alterations in both HPA axis[564,569,570] and NA system functioning[569,571] have been reported to alter performance in tests of anxiety, but no such alterations were found in either experiment 3 or 4. One possibility is that alterations anxiety-like behavior habituate quickly, disappearing after a few days of repeated administration. Secondly, this hypothesis does not propose a ready answer for why these effects, if existing, were not maintained after washout (experiment 2). While it is possible that a return to baseline would happen after sufficient washout time, it seems unlikely that such an effect would leave no lasting traces, especially when administration occurs during such a critical neurodevelopmental period as adolescence. This latter point, however, should be qualified by the fact that, in the only previous study of chronic adolescent HU-210 exposure[321], females were found to not have lasting alterations in stress reactivity, whereas an increase in this parameter was found in males. Given that, in the present work, female rats were used, this may help explain why we found no lasting alterations. Nonetheless, it must be noted that our findings are still in contradiction with the reports suggesting both rodent and human females to be more susceptible to the deleterious effects of chronic adolescent CBRA exposure.

Another putative explanation for the contrasting results of experiments 3 and 4, focuses on the role of the lateral habenula (LHb) in depressive-like behavior[572]. Specifically, this brain region is known for having a strong modulatory influence over reward circuitry, and for being involved in the emission of

aversive and avoidance responses to stressful stimuli[572]. In line with this, the LHb has been found to be overactivated in animal models of depression[573,574], and in humans suffering from depressive disorders[575].

Critically, the ECS has been shown to have a key role in the regulation of LHb activity[576], and manipulations of habenular ECS functioning have been demonstrated to lead to alterations in stress-coping behavior[516]. Specifically, direct LHb microinfusion of CB_1R antagonist/inverse agonist rimonabant or non-selective CBRA WIN 55,212-2 have been shown to lead to increases in active or passive stress-coping strategies, respectively. Moreover, a recent study from our lab[54], where adult mice were chronically exposed to WIN 55,212-2, found both habenular metabolism and functional connectivity with other brain regions (including those of the mesolimbic reward circuitry) to be altered as a result of treatment.

Thus, it may be the case that chronic adolescent exposure to HU-210 led to a dysregulation of adequate habenular functioning, by disrupting the ECS functioning in this region. One possible mechanism for this would be predicated on a downregulation and/or desensitization of CB_1R, leading to altered LHb excitability, which would modulate both reward and stress-coping circuitry. Regarding reward functioning, HU-210 exposure would increase LHb excitatory output over the inhibitory interneurons modulating the VTA, by impairing ECS functioning. This increased inhibitory tone over DA signaling would then lead to decreased sucrose preference/intake due to impaired reward functioning. Regarding stress-coping behavior, the downregulation and/or desensitization of lateral habenular CB_1R would translate itself into an effect functionally equivalent to that observed after rimonabant LHb microinfusion. Curiously, in the work of Berger et al.[516], a gender-dependent difference was found in this region, such that acute rimonabant microinfusion led to similar antidepressant-like effects in both genders, but acute microinfusion of WIN 55,212-2 led to prodepressant-like effects only in male rats. This could contribute to explain why, in experiment 2, no differences were found in the mFST.

Importantly, this explanation does not necessarily preclude the former one, as both stress hormones[577] and NA[578,579] have been shown to have powerful modulatory influences over LHb functioning. Indeed, the LHb receives direct excitatory input from the paraventricular nucleus (PVN) of the hypothalamus[572], a region known to be both the main locus of CRF synthesis and release, and strongly modulated by the ECS[551]. Furthermore, CRF has been shown to markedly increase LHb excitability through the modulation of eCB signaling, opening up the possibility for an interaction between HU-210-induced increases in CRF

and LHb ECS dysfunction[577]. In addition, NA is known to be released in the LHb, such that increased NA levels lead to increases in anxiety-like behaviors[578].

Like the previous explanation, this hypothesis has two limitations: for one, it does not adequately explain why no alterations were observed at the level of anxiety-like behavior. While habituation may happen more rapidly for this dimension of affective functioning, than it does for either stress-coping or reward functioning, this is nonetheless a question deserving further study. Secondly, this hypothesis does not propose a clear explanation for why alterations did not persist into adulthood after HU-210 administration, when that is the case for other CBRAs.

A third possible explanation, for the contradictory results of experiments 3 and 4, is that it might indeed be the case that HU-210 simply induces opposing effects on different dimensions of affective functioning. While this is somewhat counterintuitive, and no mechanistic explanation can be readily postulated for it, it is possible that HU-210 treatment may positively modulate some aspects of stress-coping behavior, while negatively modulating reward functioning which, despite having some overlap, are likely underpinned by different brain circuitry.

2 – Future Perspectives

The overarching aim of the present work was to characterize the effects of chronic adolescent exposure to HU-210 on both anxiety and depression related behavioral outcomes, as well as on one of the key molecular components of the ECS, the CB_1R. To this end four experiments were performed, such that the results of one experiment informed the design and hypothesis of the succeeding ones, in an effort to create a full and comprehensive account of what was observed at each step.

In summary, data points to the possibility that HU-210 may be a pharmacologically unique CBRA. Indeed, despite its much increased potency, efficacy and affinity for CBRs (and in special for CB_1R) in comparison to previously studied CBRAs, treatment with HU-210 had no apparent long-term effects on any of the outcomes assessed. Thus, as the current work stands, the overall derivable conclusion is that, contrasting with other CBRAs, chronic adolescent exposure to HU-210 does not induce lasting deleterious effects on either anxiety- or depressive-like behaviors. Moreover, when testing was performed in close proximity to the end of the drug administration period, the pattern of results obtained was entirely self-

contradictory, with both antidepressant- and prodepressant-like responses being observed in two different tests of depressive-like behavior. As such, it seems HU-210 is not incapable of altering affective functioning in adolescent animals, but that whatever alterations it does induce are largely abated, if enough time is allowed for. This finding, if confirmed, would bode well for the future of cannabinoid-based therapeutics.

The results obtained in the present work leave open a plethora of questions and experiments to be performed. For one, the overall picture obtained from this work suggests that HU-210 has pharmacological properties that set it apart from other CBRAs, leading to its differing effects on long-term affective behavioral changes. This finding may have critical implications for the cannabinoid-related literature, since it suggests a need for caution when drawing conclusions based on data obtained with different cannabinoid-related drugs. The present work also points towards an even more evident flaw in the literature: that researchers, ostensively testing the same hypothesis (in this case long-term affective behavioral alterations induced by chronic adolescent exposure to CBRAs) will use numerous different administration and testing protocols – and variations on those protocols – to do so. Indeed, this lack of methodological homogeneity, while sometimes needed, may be masking differences in actual drug effects. Thus, one interesting future avenue of work will be the comparative study of the most commonly used non-selective CBRAs, with regard to their long-term effects. In line with this, we are already designing a set of experiments where different groups of animals will be administered equipotent doses of different CBRAs (i.e., THC, HU-210, WIN 55,212-2 and CP 55,940), using a protocol similar to that used in experiment 2. Testing will be performed using the same test battery used here, after a 30-day washout period. Moreover, to complement these results, molecular analyses and ex-vivo electrophysiological recordings, as well as behavioral tests of cognitive function, will also be performed. The combined results of this set of experiments will, then, allow for a more solid conclusion of whether there are indeed differences between HU-210 and other CBRAs, that underlie their long-term effects upon affective behavior.

As stated before, these differences, should they actually exist, may result from either ECS or non-ECS interactions. As a starting point to disentangle these possibilities, one important step would be the identification of the role that CB_1R has in the long-term effects of HU-210. This is, at first glance, a technically easy task to perform, as one could simply co-administer HU-210 with a selective CB_1R

antagonist, to determine how this modulates the outcomes observed. However, when focusing on affective behavior, this is not as simple as it appears, given that there is ample evidence that CB_1R antagonists are themselves prodepressant[515], and may, thus, bias the results. One possible workaround this problem would entail the use of newer generation CB_1R neutral antagonists, instead of the commonly used CB_1R antagonists – such as rimonabant or AM251– which may actually be inverse agonists[580–582]. While novel drugs, such as NESS0327[583] and AM4113[584,585], do not share this characteristic, and may not have an impact on affective functioning, this possibility has yet to be adequately studied. Likewise, it would be interesting to study the downstream effects of CB_1R activation by HU-210. Specifically, given that there is evidence of biased agonism occurring with other CBRAs[391], it would be critical to determine exactly what G-protein subtypes are activated as a result of CB_1R binding HU-210.

To assess the off-target actions of HU-210, large scale receptor binding assays would be the most obvious and effective tool for the task. This, although resource- and technically-intensive, would narrow down the list of possible targets to be further investigated as possible sources for the differences between the actions of HU-210 and other CBRAs.

Paralleling this line of study, other avenues for further research are also interesting pursuits. For one, given the contradictory nature of the behavioral findings observed when testing was performed soon after the end of drug administration, additional behavioral tests should be performed to further assess anxiety- and depressive-like behaviors. In this regard, an experiment could be performed where animals are tested in both the SIT and the MBT, soon after the last HU-210 administration. Similarly, other tests not used here, such as the intracranial self-stimulation paradigm, or the LDBT, could also be performed in similar time-frames, so as to provide further measures of these behaviors. Moreover, given that HU-210 may be inducing locomotor alterations not detected by the OFT, homecage activity recordings would be indispensable in future works. Furthermore, given that, in the present work, the short-term effects of HU-210 exposure on the mFST and SPT were never tested in the same series of animals, it would be interesting to do so, so as to preclude the possibility of the contrasting results observed stemming from differences in the tested animals.

In addition, given that western blotting is not a quantitative technique for the assessment of receptor levels, radioligand binding studies could also be performed to determine if/how CB_1R density (at both adolescence and adulthood) is altered by adolescent exposure to HU-210.

Relatedly, given that the effects of chronic adolescent exposure to HU-210 were observed in the short-, but not in the long-term, and that the effects of treatment on weight gain are normalized 15 days after the last administration, it would be interesting to test whether the behavioral alterations observed show similar progression. Thus, testing could be performed after a 10-day washout, allowing for the full battery of behavioral tests to be performed. Results from this experiment would further help determine if the lack of lasting effects observed here is, in fact, related to a washout of drug action.

Similarly, given that prodepressant- and antidepressant-like responses are known to be associated with decreased[398,399] and increased hippocampal neurogenesis[586], respectively, this should also be assessed, in relation to the results obtained in experiments 3 and 4. This work is already underway, such that animals were injected with 5-bromo-2'-deoxyuridine (BrdU), a synthetic analog of thymidine, allowing for the immunohistochemical evaluation of whether adolescent treatment with HU-210 leads to alterations at the level of both neural stem cell proliferation, as well as at the level of their differentiation into neuronal cells. As such, finding an antineurogenic effect would support the idea that the behavioral results of experiment 3 are misleading – and that adolescent HU-210 leads to a prodepressant-like effect – whereas finding the opposite effect would lend credence to the results of experiment 3, suggesting HU-210 treatment to be antidepressant-like.

As one of the hypothesized mechanisms behind the contrasting results observed when animals were tested immediately after the end of drug administration, concerns the interaction between the HPA axis and the NA system, numerous experiments can be performed to test this hypothesis. For one, the protein levels of both glucocorticoid and adrenergic receptors could be assessed in several regions of interest, such as the hippocampus, the PFC and the hypothalamus. Likewise, the levels of circulating NA and stress hormones could be assessed in blood and/or CSF samples of animals, across time and drug administration, to determine how continued drug exposure affects these parameters. In addition, pharmacological studies could be carried out to test some assumptions: for example, glucocorticoid receptor antagonists could be administered concomitantly with HU-210, to determine if the contradictory results observed in

experiments 3 and 4 would remain similar, or were somehow modified – thus allowing for a better understanding of the role that HPA axis activity may have on the behavioral phenotype observed here.

Finally, given that one of the proposed explanations for the contradictory findings in experiments 3 and 4, involves the role of the ECS in the LHb in the control of affective behavior, this line of investigation would also be an interesting one to follow. As a first step, immunohistochemistry could be used to assess the expression of several key ECS components in this region, namely of CB_1R and CB_2R, degradative enzymes FAAH and MAGL, as well as of the eCBs AEA and 2-AG. Alterations in these ECS components would provide guidance for further studies into the role that the lateral habenular ECS may play in affective behavior, and in the alterations observed here. Moreover, since previous evidence suggests that both stress hormones and NA have modulatory effects over LHb excitability, the effects of HU-210 exposure on the habenular expression of elements of these systems (such as glucocorticoid and adrenergic receptors) could be assessed by similar methodology, as a first step in understanding what – if any – role they play in the effects herein observed.

3 – Conclusions

The present work suggests three primary conclusions. First, results show a clear difference between HU-210 and other CBRAs, with regards to its long-term effects on affective behavior, suggesting some pharmacological differences not yet described. Secondly, that no long-term effects were observed after a period of chronic adolescent administration, suggests that HU-210 may prove to be an interesting candidate for future therapeutic research. Moreover, that differences between drugs of the same class can lead to such marked differences in outcomes, suggests that this should be a topic of investigation, for the future development of cannabinoid-based therapeutics. Finally, the contrasting results obtained in the latter two experiments suggest that, similar to what is observed in human research, different dimensions of depressive-like behavior exist in preclinical animal models. As such, this underscores both the need for the use of behavioral testing, that captures as many of those dimensions as possible, so as to provide a more complete picture of the effects of any given manipulation, and the notion that – even in those ideal conditions – preclinical testing may not fully reproduce or mimic the findings observed in humans.

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